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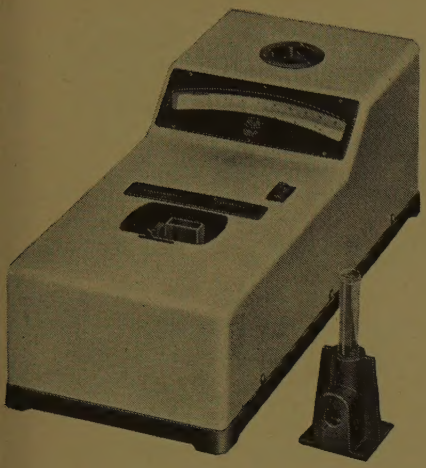

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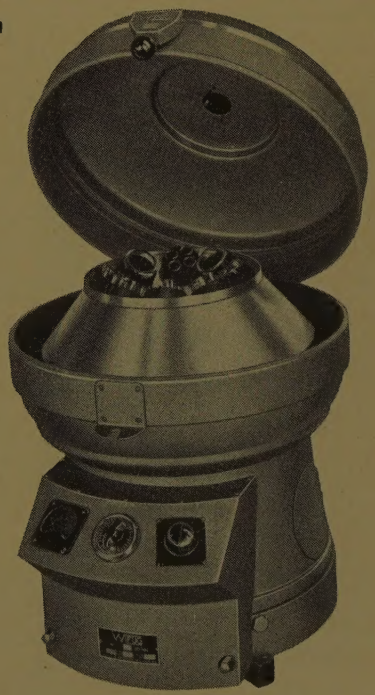

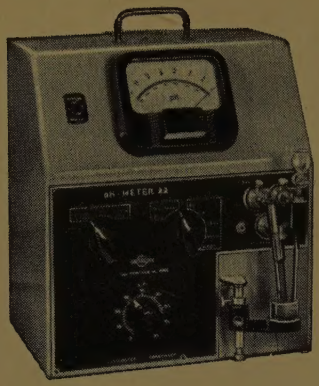

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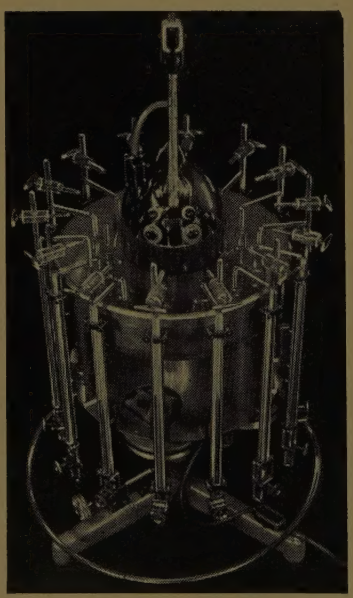

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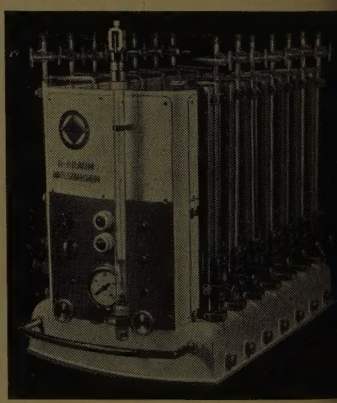

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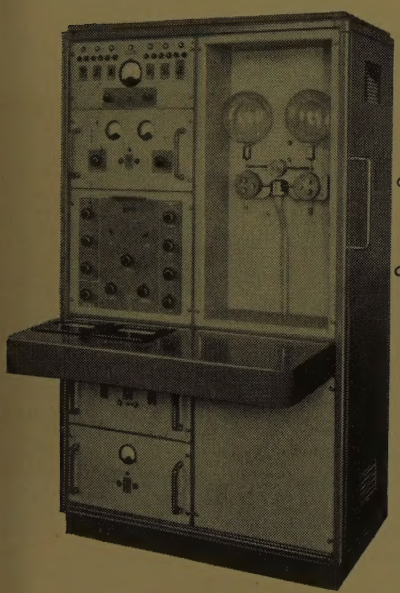

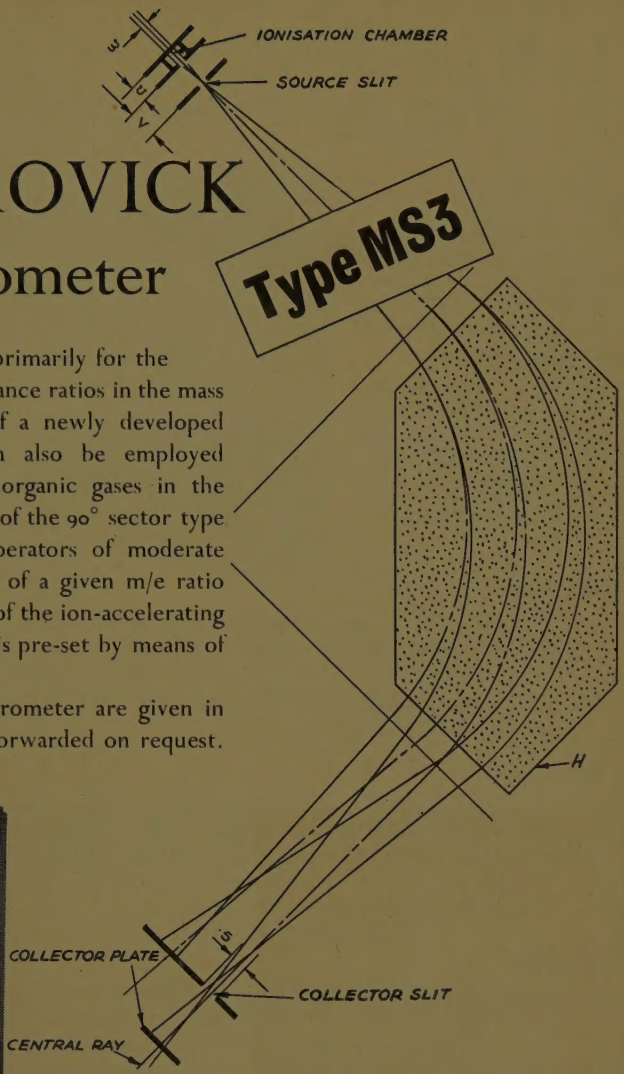

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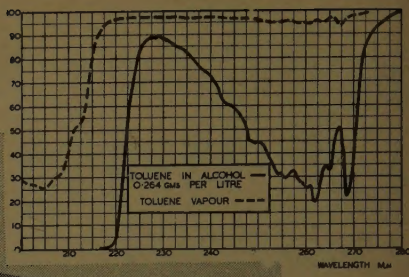

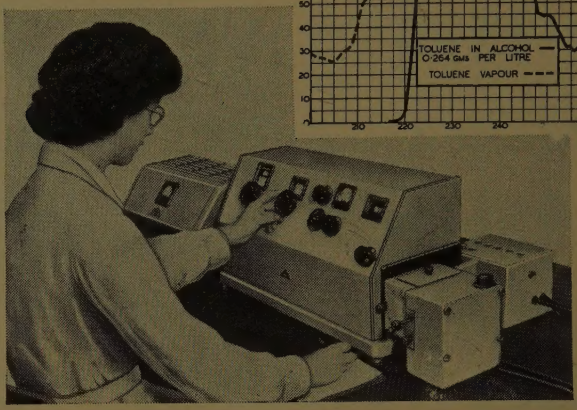

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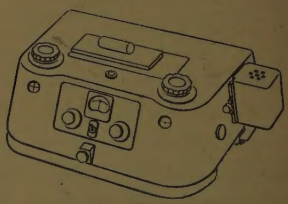

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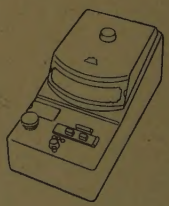

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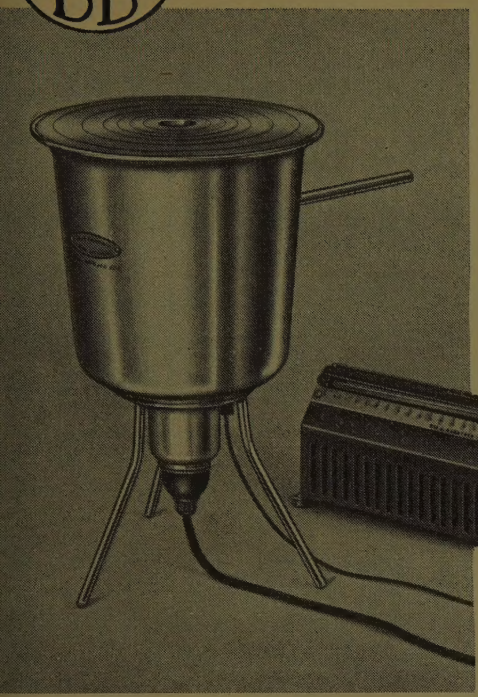

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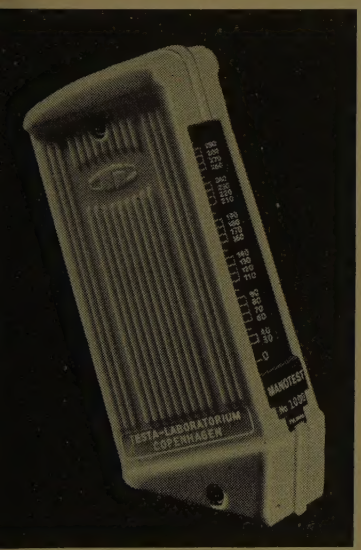

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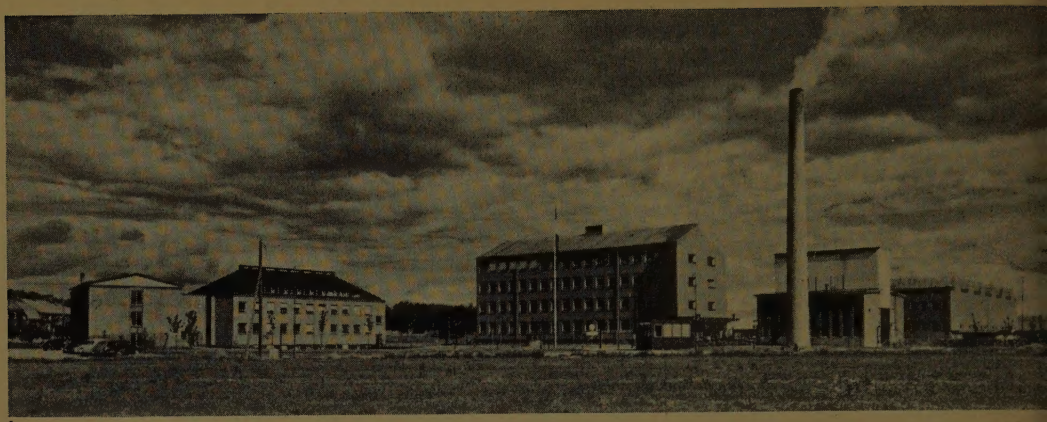

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THE ANTIPNEUMOLYSIN REACTION AND ITS CLINICAL APPLICATION

By GÖSTA TUNEVALL

From the Municipal Bacteriological Central Laboratory, Stockholm
(Head: Prof. H. Davide, M.D.)

(Received for publication August 22, 1952)

Antipneumococcal immunization has been ensively studied with methods such as ts for agglutinins, precipitins, tropins, mplement-fixing, capsular-swelling, and use-protective antibodies. When applied children below two to three years of age, se methods have with few exceptions rlens 1943) failed to disclose any evidence immunization following pneumococcal in- ion (Gundel & Schaefer 1931, Clausen & ber 1941) or injection of pneumococcal cines (Hodes, Ziegler, & Zepp 1944, lin 1949 a).

The antilysin reactions have proved in- pensable tools for the study of strepto- cal and staphylococcal infections and the t reactions to these bacteria. For the estigation of the common infections of dhood, which to a very great extent are to pneumococci (Eckert-Möbius 1927, lin 1949, Bjuggren & Tunevall 1952, nevall 1952), a corresponding method, the ipneumolysin reaction, should be valuable. The basis for this reaction is well established, as pneumococci are known to form a haemolysin which is soluble and antigenic, common to the species, and largely specific to it. With few exceptions the haemolysin has been found in all pnumococcal types tested. It has proved to be a protein of high molecular weight reversibly or, on more vigorous oxidation, irreversibly inactivated by oxygen, thermolabile, and blocked by minute amounts of lipids e. g. cholesterol. Previous investigations of pneumolysin were reviewed by Oker-Blom (1948).

The first attempts to demonstrate antipneumolysin formation in pneumococcus pneumonia failed (Cole 1914, Cotoni & Chambrin 1928) but later studies resulted in the recording of high antilysin rates (Todd 1934). An antipneumolysin reaction for clinical use was evolved by Oker-Blom (1948) who performed antipneumolysin titrations in healthy adults and in pneumonia patients, recording increased antipneumolysin rates in 85 per cent of lobar pneumonia cases.

The present investigation

In connexion with a study of oto-rhinological infections in children (Bjuggren & Tunevall 1952, Tunevall 1952) a method for routine determinations of antipneumolysin (APl), founded largely on the same principles as the procedure used by Oker-Blom (1948), was evolved. The present report deals with some observations concerning the technique and the standardization of the reaction, as well as its specificity. Further, the results of APl tests in healthy adults and children, and the reaction of children suffering from pneumococcal infections are reported.

EXPERIMENTAL

THE PREPARATION OF PNEUMOLYSIN

Of media previously found suitable for pneumolysin production (Mörch 1946, Oker-Blom 1948) ox heart broth of the type used for streptolysin preparation (Ipsen 1944) was chosen as readily available and, according to preliminary tests, somewhat superior to other routine media. It was enriched with 1 per cent horse serum.

A Type 6 B pneumococcus strain found to be a good haemolysin producer was regularly used. Prior to inoculation of the pneumolysin broth the strain was repeatedly subcultured in 5 per cent serum broth at about 8 hours' intervals.

500 ml lots of ox heart broth in 3 l. Erlenmeyer flasks were inoculated with 5 ml of a 6 hours old pneumococcus culture and incubated aerobically at 37° C for 14 hours. At the end of this period the phase of logarithmic growth should be passed and the pneumolysin content of the culture should just have reached its maximum. After cent fugation the supernate was Seitzfilter whereby the first 200 ml were discarded, a the remainder was kept *in-vacuo* for 1 ho 0.1 per cent sodium hydrosulphite was ad and the vacuum maintained for another h hour. The pneumolysin was preserved un liquid paraffin at 4° C.

The potency of the pneumolysin was termined as proposed by Oker-Blom (19 and expressed in terms of MHD (minim haemolytic dose). The pneumolysin serially diluted twofold in normal sa in 1 ml amounts and 0.5 ml of a 2 per c thrice washed sheep erytrocyte suspens was added to each tube. As MHD was corded the least amount of pneumoly effecting complete haemolysis in 45 mint at 37° C. As a rule the MHD of the pn molysin lots used was 0.008 ml.

THE ASSAY OF ANTIPNEUMOLYSI

The serum to be tested was diluted normal saline 1 : 25 (or, for special purpo less) and further serially twofold in 0.5 amounts. To each tube one *test dose* of pn molysin, diluted to the volume of 0.5 was added. As test dose 16 MHD, or ally about 0.125 ml, was used, this being least amount which displayed clear-cut points of the inhibition of haemolysis. A incubation for 15 minutes in a water at 37° C, 0.5 ml of a 2 per cent th washed sheep erythrocyte suspension added to each tube. A new incubation 45 minutes at 37° was performed, and tubes were then kept in the refrigerator o night, to be read on the following day.

The calculation of the antipneumolysin ntent was done on the basis of a 50 per cent aemolysis reading (Packalén & Bergqvist 947) of the test series, under comparison ith a standard series identically prepared ith a reference serum.

Initially a Pneumococcus Type 6 B rabbit mmune serum was used for reference. On epeated determinations it was found to cause 0 per cent inhibition of haemolysis by 16 IHD in a dilution of 1 :400. Arbitrarily it as considered to contain 400 antipneumo-sin units (AU) per ml. Later on a human erum (G.A.) containing 560 units was used or reference.

It may be mentioned that Oker-Blom 1948), working with a test dose of 25 MHD, btained titers, the inverse values of which ere likely to correspond to about ⅔ of the alue expressed by the unit chosen in the resent investigation. A direct comparison f the two standards was not possible, but he assumption of a ⅔ ratio was supported y the results of parallel titrations with 16 nd 25 MHD. A comparison of the titer istributions in healthy adults also corro-orated the same evaluation (Oker-Blom 2 per cent $\geqq 400$, Tunevall 17 per cent ≥ 500). However this comparison cannot e other than approximate, as the reading nd calculation methods were different.

The accuracy of the antipneumolysin titrations

To check the accuracy of the method, three uman sera were titrated repeatedly during he course of the work, 20, 10, and 8 times espectively. The mean error for every serum was calculated by inserting the values obtained into the formula (Oker-Blom 1950)

$$\sigma (\log p) = \sqrt{\frac{\Sigma (\log xp - \log mp)^2}{k - 1}},$$

where σ p is the mean error for every serum, xp the API values for each serum, mp the mean of the determinations, and k the number of determinations for every serum. The mean errors thus obtained for the three sera were $-26 + 35$ per cent, $-14 + 16$ per cent, and $-23 + 30$ per cent, respectively.

Further, 30 sera were titrated on two consecutive days. For these determinations the mean error was calculated according to the formula (Lagercrantz 1948):

$$\sigma (\log a) = \sqrt{\frac{\Sigma (\log x - \log y)^2}{2n - 1}},$$

where σ a is the mean error for the determinations, x and y the two values obtained for every serum, and n the number of sera. The mean error obtained was $-26 + 35$ per cent.

The joint mean error for all series was $-24 + 32$ per cent, which is close to the corresponding figure found for antistreptolysin determinations (Lagercrantz 1948, Oker-Blom 1950). All titrations were performed by the same technician.

THE SPECIFICITY OF THE ANTIPNEUMOLYSIN REACTION

The antihaemolysin formed in animals on pneumolysin injection has been found to react with, apart from pneumolysin, *B. perfringens* haemolysin, though only feebly (Cotoni & Chambrin 1928). Of more prac-

tical significance was the observation that antistreptolysin hyperimmune horse serum exerted a neutralizing effect on pneumolysin, averaging to one tenth of its action on streptolysin (Todd 1934). As the clinical material studied in connexion with the present investigation presented simultaneous increases of the APl and the AS rates, it was endeavoured to ascertain the degree of antigenic overlapping between pneumolysin and streptolysin as well as the degree of cross-reaction between the two lysin-antilysin systems.

Table 1a. *The antihaemolysin reactions obtained with antipneumococcal and antistreptococcal rabbit immune sera.*

	Antipneumolysin titer	Antistreptolysin titer
Antipneumococcal sera		
Type 11 A	1280	36
» 24	3600	110
» 35 B	640	≦ 36
Antistreptococcal sera		
Type 4	≦ 18	900
» 8	36	1100
» 22	80	7200

Immunization experiments

Three antipneumococcal (Types 11A, 24, 35 B) and three antistreptococcal (Types 4, 8, 22) diagnostic rabbit sera were tested for their Apl and AS content, with the results given in Table 1 a. Heterologous antilysin titers occurred in sera with a very high homologous antilysin content, in conformity with the observation of Todd (1934) mentioned above. These non-specific reactions, however, were very feeble in comparison to the high homologous antilysin titer.

Table 1b. *The antihaemolysin reaction obtained with antipneumolysin and ant streptolysin Guinea pig immune sera.*

	Antipneumolysin titer	Antistrepto-lysin titer
Antipneumolysin sera		
1.	560	≦ 25
2.	800	≦ 25
3.	560	≦ 25
Antistreptolysin sera		
4.	≦ 18	140
5.	25	280
6.	≦ 18	70

Table 2. *Results of neutralization experiments with sera from Guinea-pigs injecte with active haemolysins.*

Antipneumolysin sera	Antipneumolysin titer			
	Before neutralization	Neutralized with		Ox hea broth control
		pneumolysin	streptolysin	
1.	100	≦ 18	50	100
2.	140	≦ 18	70	-
3.	100	≦ 18	70	-
Antistreptolysin sera	**Antistreptolysin titer**			
4.	140	70	≦ 25	200
5.	280	200	36	-
6.	70	36	≦ 25	-

Further, three guinea-pigs were inject subcutaneously thrice a week with pneum lysin (MHD 0.008) in doses of 0.2 m 0.4 ml and 0.5 ml, the last amount bei given ten times. Three other guinea-pi were given the same doses of streptolys (MHD 0.008). One week after the l injection the animals were bled and the se

ed for their API and AS content. The ılts, shown in Table 1 b, were similar to se obtained with the rabbit sera.

Neutralization experiments

The sera from the guinea-pigs of the pre- us experiment were subjected to the fol- ing neutralization experiments, arranged done by Packalén (1948). Serum was ıted in normal saline (if necessary) to tain about 100 AU/ml and subsequently 25 in undiluted active haemolysin broth rates. The mixture was incubated for minutes at 37° C for neutralization, and ther for 45 minutes at 56° C for inactiva- ı of remaining free haemolysin. Finally, mixture was tested for persisting anti- molytic activity. Controls in which the molysins were replaced by filtered and uced ox heart broth were run parallell. As will be seen from Table 2, homologous ıtralization was complete or almost com- te. A partial neutralization took place also ween heterologous reactants, whereas the th controls were negative. Apparently, heterologous reaction, which was feeble the routine tests described above, was oured by the higher concentration of the molysins present in the neutralization s.

THE ANTIPNEUMOLYSIN CONTENT OF HUMAN SERA

Healthy adults

For the evaluation of API rates obtained healthy children of different ages and in ldren suffering from pneumococcal in- ions, it was considered necessary to know average API content of sera from healthy ılts. Hence, 300 subjectively healthy per- sons not known to have suffered from infec- tious diseases during the preceding two or three months (170 blood donors, 60 persons applying for appointments within a commer- cial organization, and 70 pregnant women admitted to a confinement clinic) were exa- mined for API. The results are reported in Figure 1. Within this group 17 per cent displayed API rates of 500 or more.

Diaplacental transfer of antipneumolysin

A comparison between the API rates of 70 mothers and the titers obtained in blood from the umbilical cord at confinement is shown in Figure 1 and 2. When the API of the mother was below 160, the conformity between the maternal and the foetal API was rather close. When the API of the mother was 160 or more, on the other hand, the API rate of the child was often below that of the mother. The difference was often con- siderable, especially when the mother's titer was very high.

Children not suffering from pneumococcal infection

The sera of 92 children below three years of age, apparently healthy or suffering from non-infectious diseases, were tested for API, with the results given in Figure 1. As some of the children were not bacteriologically examined and the age groups to which they were assigned were small, no differentiation between pneumococcal carriers and non- carriers was undertaken.

Further, sera from 120 children from three to seven years of age were tested. 108 of these children were comprised in a report published elsewhere (Tunevall 1952) and

dealing with the incidence of maxillary sinusitis in children. All children were apparently healthy. They were examined thrice at about two months' intervals, with nose cultures, X-ray of the maxillary sinus and, if sinus changes were found, with sinus puncture and cultures from the discharge. On all three occasions their API was determined. In non-carriers the last API rate was counted, in nasal carriers and children harbouring pneumococci in the maxillary sinus the titer found on the last occasion when pneumococci were isolated from the child was recorded. The results are given in Figure 1.

Measurable API amounts were generally present during the first two months after birth, but absent during the subsequent three months. From the sixth month onwards detectable immunization were again common, though very low rates were not infrequent during the rest of the first year and also during the second year of life. Already by the third year the titer distribution was similar to that of adults, titers of 500 or more amounting to about 20 per cent. The prevalence of high titers was still more pronounced in the 3—7 years group. Even in

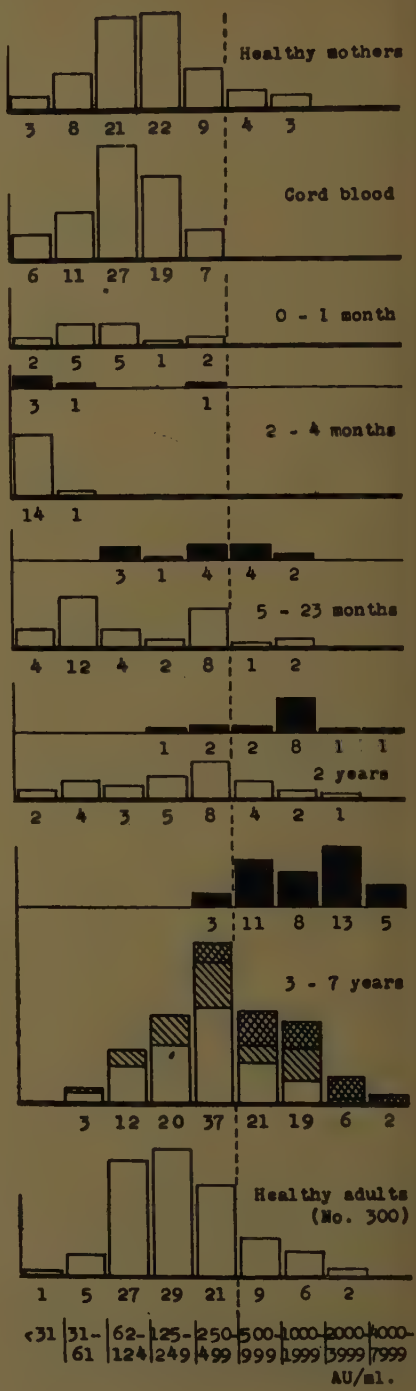


Fig. 1. Antipneumolysin titers of healthy mothers and cord blood, of healthy children, and children suffering from pneumococcal otitis, as well as in healthy adults.

Legend to Figure 1

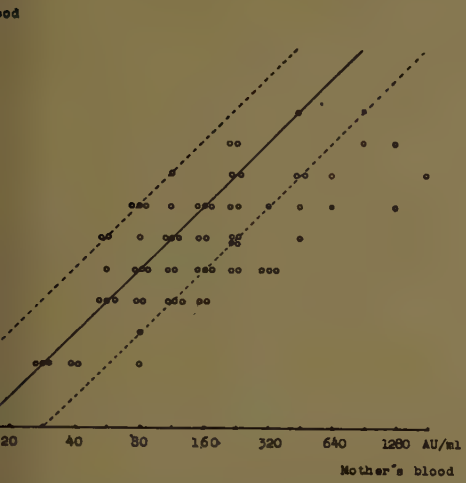
Highest titer reached in pneumococcal otitis

Titer of children harbouring pneumococci in paranasal cavities

Titer of nasal carriers

Titer of apparently healthy subjects (in the 3 - 7 years group non-carriers)

Figure at bottom of column denotes the number of subjects (in the group of healthy adults given in percent)



2. Comparison of the antipneumolysin rates of healthy mothers and the titers obtained in blood from the umbilical cord.

non-carrier group 22 per cent of the dren presented API rates of 500 or more. he »unstripped« group the corresponding re was 40 per cent.

Within the 3—7 years group the tendency pneumococcal carriers towards higher rs than those found in non-carriers could be statistically verified unless the pneu-cocci were recovered also from the para-al cavities, in which case the deviation almost significant statistically ($P<0.01$).

he reactions to pneumococcal infections.

The API titers observed in children during course of a pneumococcal otitis are wn in Figure 3. The API curves of children could not always be followed n the beginning of the infection, which en had lasted for some time when the ic suppuration started. Further, the reac-is varied considerably as to length and ght, also within each of the separate age ups. However, if the means of all titers

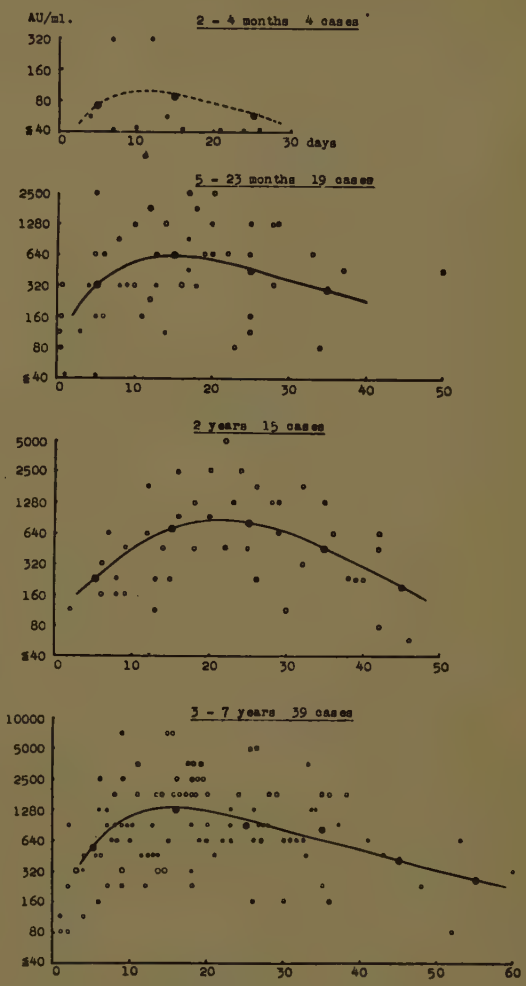


Fig. 3. Antipneumolysin titers of children during the course of pneumococcal otitis. The mean titers of every 10 day period (large black points) are connected to "average" curves.

obtained during each 10-day period of the disease were calculated separately, they were found to coincide with a curve which may be regarded as representing the average reaction of the age group. At this comparison the disease was considered to begin already at the start of the upper respiratory infection

often preceding the otitis. Further, if more than one value was obtained from a child during the same 10 days period, only the first of them was recorded.

The 2—4 months group, the mean curve of which was drawn merely for comparison, presented four cases with no or very feeble API reactions. Already from the age of 5 months onwards the reactions were as a rule lively and presented a character similar to that displayed within higher age groups. However, as will be seen from Figure 1, the older children on an average reached higher maximum titers.

SUMMARY AND CONCLUSIONS

Pneumolysin preparations appropriate for antipneumolysin (API) determinations could be obtained by filtration and reduction of pneumococcus cultures in the usual ox heart broth used for streptolysin production. The API content of serum could be determined by a simple procedure in all essentials similar to the methods used for antistreptolysin and antistaphylolysin determinations. The accuracy of the determinations was not inferior to that previously found for antistreptolysin titrations.

No significant neutralization of pneumolysin by antistreptococcal or antistreptolysin immune sera, or of streptolysin by antipneumococcal or antipneumolysin sera was observed. Very high-titered sera, however, gave slight reactions also with the heterologous haemolysin, as observed previously with antistreptolysin hyper-immune serum versus pneumolysin. This partial overlapping between the two lysin-antilysin systems was more evident in neutralization experiments, where the concentration of haemolysin was higher than in the tubes of a routine ar pneumolysin or antistreptolysin test.

Thus, the simultaneous API and antistr tolysin increases not infrequently obser in the clinical material studied by the aut were probably not due to overlapping re tions. The possibility of so-called anamne reactions should be borne in mind in t connexion, though double infections, be common in the series, provided a satisfact explanation in most cases of simultane elevation of more than one antibody titer

API determinations in 300 healthy ad indicated that, with the standard unit cho an API value of 500 ought to correspond the value 200 generally accepted as the li for normal variation of antistreptoly rates.

As previously found for antistreptoly and antistaphylolysin, the API rates in bl from the umbilical cord kept close to th of the mothers, provided however, that API of the mother was low or modera high. When the mother's API was high, titer of the child often lagged consider behind.

Measurable API amounts were found infants during the first two months a birth, but already by the third month reaction was generally negative, which analogy with previous findings as to o antibodies was likely to indicate an elim tion of diaplacentally transferred API. F the age of five months API was again fo in most children and already during the t year of life the titer distribution was similar to that found in adults. In children from three to seven years of the main titer level was even higher in adults.

This fairly rapid adjustment of the mean ter level of children to the level found in lults was paralleled by the high frequency pneumococcal infections in infants and nall children observed in the clinical part the investigation, as well as the fact that e APl responses on pneumococcal in- ctions were found lively already from the ge of five months.

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THE DIURNAL VARIATION OF THE SERUM IRON CONCENTRATION

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A diurnal variation in the concentration of the serum iron with higher recordings in the morning than in the evening was first observed by Vahlquist (1941) and has since been confirmed by many research workers in this field (Hemmeler 1944, 1951, Ferroni & Indovina 1947, Nilsson 1948, Rustung 1949, Schäfer 1949, Hamilton *et al.* 1950 and others). In nightworkers the rhythmic fluctuation is reversed and less pronounced (Høyer 1944, Waldenström 1946, Hemmeler 1951).

Hemmeler (1951) has also shown that the morning values are markedly influenced by the duration of sleep the night before. Morning values recorded after a long, quiet sleep are higher than those noted after a short, or unquiet sleep. The concentration of the serum iron after a good night's rest is usually about 30 per cent higher than the evening level. Short, unquiet sleep at night diminishes this difference, and then the morning recordings can even be lower than the evening level. Work or rest during the daytime has no influence on the diurnal variation.

After a good night's sleep the highest and lowest concentrations usually occur at 8—9 a.m. and 5—8 p.m. respectively (Hamilton *et al.* 1950, Hemmeler 1951). As a working hypothesis Hemmeler (1951) suggested that the serum iron concentration is regulated b the autonomic nervous system and that th parasympathetic nervous system tends t increase, and the sympathetic system t decrease, the serum iron level.

Hamilton *et al.* (950) found no relatio ship between the adrenocortical function an the diurnal variation of the serum iro values. Thedering (1949) expressed th opinion that the serum iron values fa during the day only in people with hig morning values, but when collecting h material he did not take into account th importance of sleep.

The lack of a satisfactory explanati for this diurnal variation of the serum iro concentration was considered to warra further investigation of the problem. Th purpose of the present study was to dete mine whether variation of the serum iro values during the day is influenced by va ation in the ratio between the amount of iro passing into and out of the plasma, which in turn dependent on the variation in the ra of the metabolism of the hemoglobin.

The concentrations of bilirubin, copp potassium, and sodium in the serum we also determined in order to trace any re tionship between possible variations in the concentrations and the rhythmic fluctuati of the serum iron concentration.

Table I. *Morning (M) and evening (E) values on some serum constituents.*

se		Serum iron µ g per cent		Total iron binding capacity µ g per cent		Serum bilirubin mg per cent		Serum copper µ g per cent		Sodium in serum mg per cent		Potassium in serum mg per cent	
		M.	E.	M.	E.	M.	E.	M.	E.	M.	E.	M.	E.
.	♀	94	76	302	300	0.75	0.32	109	122	345	340	19.6	19.3
.	♀	127	82	307	312	0.67	0.28	128	120	335	332	16.4	16.4
.	♀	43	36	334	334	0.25	0.22	129	149	338	335	18.5	18.2
.	♂	149	99	321	336	0.80	0.44	133	147	342	342	17.2	18.0
.	♂	125	89	338	332	0.99	0.61	106	109	-	-	-	-
.	♀	165	136	339	350	0.45	0.21	69	67	338	338	15.8	15.8
.	♀	125	55	294	298	0.48	0.23	144	155	340	342	17.0	16.4
.	♂	195	89	340	350	1.26	0.49	110	119	338	335	15.6	14.8
.	♂	138	123	379	366	0.70	0.38	113	103	338	338	15.8	15.0
.	♂	121	100	325	325	0.61	0.51	129	129	343	343	16.4	17.6
n values		128	89	328	330	0.70	0.37	117	122	340	338	16.9	16.8

MATERIAL

Blood specimens (about 30 ml) were drawn at .m. and at 5 p.m. All of the donors had slept) hours the night before.

METHODS AND ANALYSIS

The serum iron concentration was determined modification of the method of Tompsett (1949), total iron-binding capacity of the serum (the centration of the transferrin)) by a modification Laurell's method C (1947), and the bilirubin l by the method of Jendrassik and Gròf. The per was measured after wet ashing with diethyl-iocarbamate, and the sodium and the potassium flame photometry.

RESULTS

The results of the analyses are sum-rized in Table I, from which it is clear t the serum iron concentrations were ularly higher in the morning than in the ning. The fall of the serum iron level ing the course of the day was in good eement with the decline found by Hem-er (1951) in a larger series. The total n-binding capacity of the serum and the sodium, potassium and copper levels were found to be the same in the morning as in the evening. The bilirubin concentration was, however, regularly higher in the morning than in the evening. The ratio between the morning and evening figures was slightly higher for the serum bilirubin than for the serum iron.

In order obtain an idea whether the morning values and the evening values represented the maximum and minimum levels respectively, as is the case with the serum iron concentration (Hemmeler 1951), the bilirubin concentration was determined at shorter intervals in 3 of the subjects. It is obvious from Table II that the morning and evening recordings were fairly representativ of the maximum and minimum levels during the 24 hours of the day.

DISCUSSION

Both the maximum level and the minimum level of the rhythmical diurnal variation of the concentration of the serum iron coincide

Tbble II. *Diurnal variations of serum iron and serum bilirubin values in thr individuals.*

Case 1			Case 2			Case 3		
Time	Serum iron μ g per cent	Serum bilirubin mg per cent	Time	Serum iron μ g per cent	Serum bilirubin mg per cent	Time	Serum iron μ g per cent	Serum bilirubi mg per
9	100	0.49	9	80	0.51	10	125	0.55
13	85	0.32	13	74	0.40	14	110	0.53
18	76	0.25	18	47	0.25	18	95	0.49
22	51	0.21	22	36	0.21	22	85	0.49
2	59	0.23	2	32	0.21	2	47	0.47
9	89	0.47	9	91	0.55	10	74	0.61

chronologically with the corresponding levels of the likewise rhythmically varying serum bilirubin concentration. As the greater part of the serum iron and of the serum bilirubin are derived from the hemoglobin released during destruction of aged red cells, it sounds reasonable to suppose that the diurnal variation of the serum iron and serum bilirubin concentration are ascribable to the variations in the different phases of hemoglobin metabolism.

Exchange between serum iron and storage iron is a fairly slow process, as is apparent, for example, from the slowness with which the serum iron level returns to normal after an initial increase following an intravenous injection of 5—10 mg iron. As the total amount of iron in the plasma is only about 4 mg and as the total daily serum iron turnover is about 28 mg (Huff *et al.* 1950), the relative constancy of the serum iron value throughout the 24 hours of the day is remarkable, because no special mechanism is apparently available to compensate variations in the concentration of the serum iron in the event of rapid changes in the amount of iron passing into and out of the blood. Since the greater part of the iron is derived fr hemoglobin catabolism (20 mg per day, H *et al.* 1950) it must be assumed that synthesis and the destruction of the he globin during the 24 hours of the day relatively well balanced against one anot The similarity found between the diu rhythm of the bilirubin and serum iron le may be explained by the assumption that rate of destruction of hemoglobin is sligh greater at night than during the day. amount of iron and of bilirubin liberated the destruction of the hemoglobin would t be slightly greater during the night t during the day. The serum iron and serum bilirubin levels are always highest the morning.

SUMMARY

Observations made in an investigation the diurnal variation of the serum iron c centration suggested that the diurnal va tions of serum iron can be explained a phenomenon secondary to the diurnal va tion of the hemoglobin metabolism. T hypothesis is supported by parallel chan in the bilirubin and serum iron values.

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NORADRENALINE AND ADRENALINE IN BLOOD AND URINE IN A CASE OF PHAEOCHROMOCYTOMA

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(Received for publication Sept. 25, 1952)

The direct demonstration of increased urinary excretion of catechol amines (adrenaline and noradrenaline) as a method for diagnosing phaeochromocytoma was first described by Engel & Euler in 1950 and has since been used with success by Goldenberg & Rapport (1951), Lund (1952) and Pekkarinen (1952).

The value of the method in the investigation of assumed phaeochromocytoma not demonstrable by ordinary methods or pharmacodynamical tests is illustrated by a case treated at the surgical department, University Hospital, Lund, in March 1952, and described below.

CASE REPORT

The patient was a 23-year-old man, who had always felt well until the autumn of 1948, since when he had suffered from gradually increasing, constant fatique, headache and dizziness. On physical exertion the symptoms increased in intensity and soon exhausted the patient, who was then obliged to lie down and rest. Sometimes such exacerbations occurred spontaneously, being attended by profuse sweating but never by palpitation, thoracic or abdominal pain, or increase in the severity of the headache or dizziness. He had been unable to work since the onset of the symptoms.

Initial transient albuminuria suggested nephritis, but as the blood pressure did not drop to normal after the albuminuria had passed off, essenti hypentension was assumed.

In order to determine whether sympathector was indicated, the patient was transferred to t neurosurgical department (Dr. Nils Lundberg) f investigation, where the following observations we made.

Blood pressure during bed rest: 170/120. Sig of hypertrophy of the left side of the heart. *Ren function:* normal (urea clearance, N.P.N. a urinary sediment). *Ocular fundi:* angiopathia hype tonica. Basal metabolic rate: + 21 per ce *Glucose tolerance test:* blood sugar rose to 220 m 100 cc within one hour and fell to resting level 2 hours. *Blood counts:* repeatedly normal exce for regularly complete absence of eosinophils.

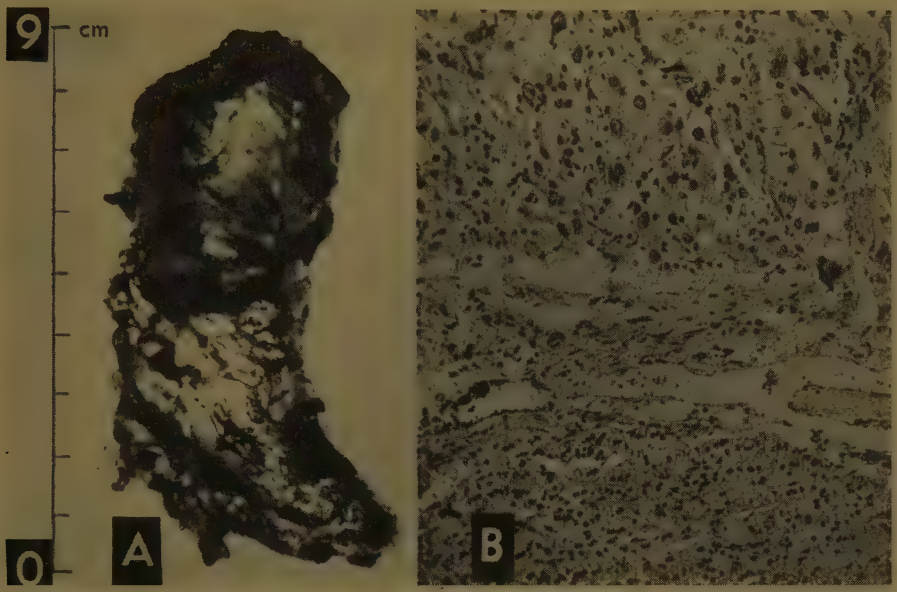
Excretion urography and X-ray investigati after retroperitoneal insufflation of air showed anomalies or signs of tumour.

Pharmacodynamical tests for phaeochrom cytoma. Tetra ethyl ammonium chloride (Etamon Astra) had no effect on the blood pressure. Regi (Ciba) produced only a slight increase in blo pressure and attendant palpation and headache.

Urinary analysis showed a greatly increas excretion of noradrenaline and thereby proved t diagnosis of phaeochromocytoma.

Operation (Ph. Sandblom). Removal of phaeochromocytoma.

The tumor was approached via a midline i cision drawn from midway between the xiphe process and the umbilicus to a point 5 cm sh of the symphysis. The left and right suprarer regions were explored. Both suprarenals were n mal to palpation and no tumor was felt. The regi of the aorta above and below the transverse me



ig. 1. Tumor from patient G. J. *A*. Tumor and left suprarenal. *B*. Microscopic picture of tumor, showing chromaffine and cortical tissue.

lon were then examined. A tumor was found teromedial to the hilus of the left kidney. The nor was the size of a hen's egg, it was rounded d firm. No other tumor was felt. Incision of e peritoneum over the tumor showed the growth be located just in front of, and adherent to, the rivascular sheath of the left renal hilus. The nor was greyish-yellow and showed some spots brighter yellow. The anterior surface was trans-rsed by a number of tortuous veins. The tumor is nourished medially by a couple of arteries the ckness of a knitting needle and a slightly larger in. There were also several small veins passing o the surroundings.

The tumor was carefully freed. It seemed to tend up into the left suprarenal, but it felt as ough it was not continuous with the suprarenal neath the cortex. In view of this relationship it emed unwise to spare the left suprarenal, for the low-gland was apparently normal. The tumor s therefore removed *en bloc* with the suprarenal. uring the dissection of the tumor the systolic pressure rose and reached a peak of 230 mm. On transsection of the veins the pressure fell to 80 mm.

Tachycardia did not occur during the operation, which the patient seemed to tolerate very well. During the latter part of the operation and afterwards noradrenaline was administered by intravenous drip.

Pathologist's report (C. G. Ahlström). The tumor (Fig. 1 A) weighed 40 gm. It consisted of cells with round or oval nuclei and with fine-grain chromatin and abundant cytoplasm (Fig. 1 B). The nuclei varied widely in size and some were monstrous. The cells were arranged in trabecular formations. Between the trabeculae were numerous vessels, especially towards the middle of the tumor, where the vessel lumina dominated the picture. Here and there the tumor cells showed a sparse deposition of brown pigment. No mitoses were observed. The tumor was covered with a thin layer of cortical tissue. It did not infiltrate the surrounding tissue. *Diagnosis* Phaeochromocytoma.

The patient made a smooth recovery and when he left hospital his blood pressure was 125/80.

Table I. *Noradrenaline and adrenaline in urine of patient G. J.*

Date	Urine	μ g per 24 hours		% Adr.	
		Adr.	Noradr.		
Febr. 26, 1952	Extract	19	1200	1.6	Before operation
	Untreated (cat's B. P.)		2400		
March 8, 1952	Extract	16	1010	1.6	»
	Untreated		1200		
April 20, 1952	Extract	1.5	34	4	After operation

Estimation of noradrenaline and of adrenaline in the blood, in the urine and in the tumor.

Urinary analysis by the method of Euler & Hellner (1951) showed a greatly increased amount of noradrenaline, while the level of adrenaline was only slightly above normal. The values recorded before and after operation are given in Table I.

The amount of noradrenaline in the urine was so large that it could be estimated by the effect of the untreated urine on the blood pressure of the cat (Fig. 2). This simple procedure is a useful screening test (Euler, 1951).

Lund & Møller (1951) have previously demonstrated the presence of increased catechol amine levels in the blood in a case of phaeochromocytoma.

Since it seemed of interest to compare the amount of noradrenaline excreted in the urine with the level in the blood, blood specimens were drawn at the beginning and end of a 1-hour period during which urine was also collected. In view of the constant hypertension of the patient, it was assum that the concentration of the blood-noradr naline was also fairly constant during su a period.

The blood catechols were estimated by t method of Lund (1951) and the urina catechols by the fluorimetric method (A. I and by the biological method (U. S. v. E

The determinations made are given Table II together with the figures obtain 5 months after the operation.

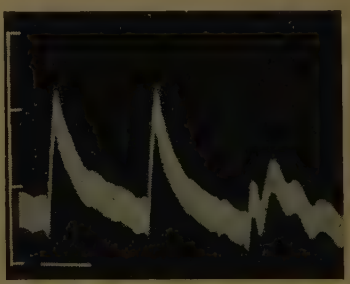


Fig. 2. Effect of 0.1 ml untreated urine of pati G. J. on cat's blood pressure compared with 0.3 l-noradrenaline and 0.4 μg l-adrenaline.

B. P. 130—190 mm Hg. Time 1 min.

Table II. *Simultaneous estimations of noradrenaline in blood and urine from patient G. J. before and after operation*

Date	Noradrenaline in urine		Volume of urine ml in 1 hour	Output of noradrenaline µ g per min.	Noradrenaline + adrenaline in blood
	fluori-metric	biologic			
3.1952 (Before op.) ..	250 µ g % (no adrenaline)	200 µ g %	44	1.8	3.4 µ g % (7.00 a. m.) 3.8 µ g % (8.00 a. m.)
8.1952 (After op.) ...	8.8 µ g % (2.1 µ g % adr.)				< 0.2 µ g %

The total amount of noradrenaline excreted with the urine during the 1-hour period was thus 110 µg as determined fluorimetrically as compared with 88 µg as measured by the biological method. Since only about 75 per cent is recovered by the procedure used in connection with the biological method the fluorimetric determination is regarded as being nearer the true value. This means that the excretion per minute in the case was 1.8 µg.

The average concentration of the blood noradrenaline was 3.6 µg/100 ml as determined by one estimation at the beginning of the period (3.4 µg/100 ml) and one at the end (3.8 µg/100 ml).

A bit of the tumor was sent to each of the two laboratories for analysis by the fluorimetric method (A. L.) and by the colorimetric and biologic methods (U. S. v.E.). The difference between the values recorded by the colorimetric and fluorimetric methods may be ascribable to an irregular distribution of the catechols in the tumor. The low adrenaline level recorded by the biological method seems to be more compatible with the low excretion of adrenalin with the urine. The possiblity that other catechol amines may have simulated adrenaline as recorded by the colorimetric and fluorimetric methods must be considered.

COMMENTS

The present case of phaeochromocytoma is of interest because ordinary diagnostic methods and pharmacodynamical tests failed to provide the diagnosis, which was only secured by estimation of the catechol amines in the urine and in the blood. The precautionary measure of urinary analysis for catechol amines before submitting a patient to sympathectomy thus revealed a phaeochromosytoma in a case in which other methods had been non informative and in which the patient had no history of the typi-

Table III. *Adrenaline and noradrenaline in tumor tissue (G. J.).*

	Adr. µg per g	Noradr. µg per g	% Adr.
Biological ..... (U. S. v. E.)	12	590	2 %
Colorimetric ..... (U. S. v. E.)	70	630	10 %
Fluorimetric..... (A. L.)	97	800	11 %

cal, but not obligatory symptoms e. g. palpitation, thoracic pain and pallor. The increased basal metabolic rate, the sweating and the hyperglycaemic response to the glucose tolerance test were, however, compatible with the condition. As is often the case, abdominal palpation and radiological investigation were also non-informative.

In addition the present case provides an interesting example of the clinical and laboratory findings that may be expected in a patient with a constant, high concentration of blood noradrenaline judged by the permanency of the increased blood pressure of the patient. This represents a condition hardly attainable in human experiments. The clinical observations commented on below suggest the occurrence of certain functional changes in the presence of such an increased blood noradrenaline.

The circulatory changes were such as may be expected in patients with a high blood noradrenaline level, i. e. increased blood pressure and normal pulse rate. The blood pressure values during bed rest were 170/120: this increase in both systolic and diastolic blood pressure is typical of the action of noradrenaline. Unlike adrenaline, noradrenaline does not produce tachycardia; therefore the absence of this symptom in the patient under discussion was also suggestive of increased blood noradrenaline.

Renal function was unimpaired, this is consistent with the observation made by recent investigation of the effect of the infusion of noradrenaline in man (Bucht, Werkö, Josephson & Ek, 1952).

A symptom of interest in the present case was sweating. It is generally believed that while the sweat glands in some animals, such as the horse, are adrenergic, the correspo ding glands in man are cholinergic. Recent however, Chalmers & Keele (1951) a Barnett (1951) showed that the injection noradrenaline into man is followed by p spiration.

As to the metabolic changes, recent inv tigations have shown that adrenaline is much more powerful stimulant than n adrenaline, the ratio of their activity bei about 5—10: 1 (for literature, Euler, 195 However, when present in sufficiently hi concentration noradrenaline may produ certain effects, and such effects should expected in the present case. This also p ved to be so. Thus the basal metabolic r was distinctly, though moderately, increas and the glucose tolerance test gave a sligh increased response.

An interesting observations was the sence of eosinophils in the blood. Humphre & Raab (1950) showed that intravenou injected noradrenaline lowers the eosinoph but is about six times less active than ad naline. In our patient the high noradrenali level apparently produced a very mark response.

Here, as usual after the experimental ministration of noradrenaline, the nerv symptoms were less characteristic. For stance, the typical feeling of anxiety fol wing injections of adrenaline was missi

The fact that the disease in the pres case was for some time believed to be ess tial hypertension is instructive since it sho how easily the two conditions may be c fused, notwithstanding the fact that the le of the blood noradrenaline may be exce vely high in one condition and not in other.

In their case of phaeochromocytoma Lund Møller (1951) found the noradrenaline ncentration in the blood plasma to be —2.4 μg/100 ml as compared with the rmal level of less than 0.2 μg/100 ml. The esent case is apparently the first to be orted in which the amounts of catechol ines in the blood and in the urine were ermined in specimens collected simultaneıly.

In the experiments reported by Euler & ft (1951) infusion of about 8 and 13 μg oradrenaline per minute increased the nary excretion by 0.20 and 0.30 μg per nute. If a similar rate of excretion (2.5 r cent of the infused amount) be assumed our case, the flow of noradrenaline into e blood stream would be $\frac{0.30}{1.8} \times 13$, or μg per minute, which is slightly more than μg/kg bodyweight per minute. This dose surely produce a pronounced increase in blood pressure and is higher than any se used by Goldenberg, Pines, Baldwin, eene & Roh (1948). Their highest dose, μg/minute/kg bodyweight produced a e of 15—50 mm Hg in the blood pressure.

SUMMARY

A case of phaeochromocytoma is deibed in which X-ray investigation and armacodynamical tests with tetraethylmonium and Regitin were non-informae. The diagnosis was established by the reased excretion of catechols with the ne (1010—2400 μg noradrenaline and —19 μg adrenaline per 24-hour period). Estimation of catechols in samples of the blood and of the urine from the same period showed an average blood level of 3.6 μg/100 ml noradrenaline and a corresponding urinary output of 110 μg per hour.

The high systolic and diastolic blood pressure, the electrocardiographic changes, the increased basal metabolic rate, the response to the glucose tolerance test, sweating, eosinopenia and fatigue are explained as symptoms and signs of hypernoradrenalinemia.

Biological estimation of the catechols in the tumor, which weighed 40 g, showed 590 μg/g noradrenaline and 12 μg/g adrenaline.

After operation the blood pressure and the level of the urinary catechols returned to normal.

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METHOD FOR RECORDING AORTIC PRESSURE PULSES[1]

By S. RADNER

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(Received for publication Oct. 6, 1952)

Aortic hemodynamics have been but little plored in man, probably because no simple thod of investigation has been available. e few studies made on human subjects lans, Katz, Graham, Gordon, Elisberg & ber, 1951, Schnabel, Fitzpatrick, Peter- Rashkind, Talley & Raphael, 1951, Rad- Cournand, Cathcart & Fishbein, to be lished.) have involved catheterization of aorta from a peripheral artery.

We have been applying a new method for purpose, which might be suitable for tine use. The arch of the aorta is punc- ed percutaneously from the suprasternal ch in the neck, and recordings of the tic pressure pulses are made through the dle.

EQUIPMENT

simple needle instrument was constructed . 1). The needle, which has an outer diameter .9 mm and an inner diameter of 0.5 mm, is nted on the straight limb of a three-way stop- . The needle is supported by a closely fitting der which surrounds its proximal 4 cm. The distal length of the needle is 10 cm. Through straight limb of the stop-cock a stilette is in- d into the needle. The transverse limb of the -cock is connected with an infusion bottle, con- taining saline-heparin solution under suitable pressure.

After the aorta has been punctured, the stilette is withdrawn and the needle connected with a Tybjærg Hansen manometer, by means of a canalized knob and a small piece of a Cournand catheter, size 6 or 7.

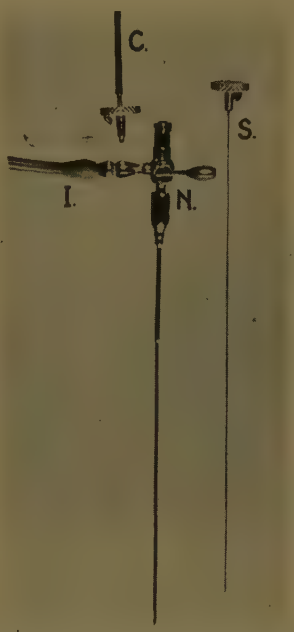

Fig. 1. Needle instrument for suprasternal puncture of the aortic arch. N = Needle with three-way stop-cock. S = Stilette with bayonet knob. I = Infusion tubing. C = Catheter with canalized bayonet knob.

[1] Presented at the Staff Meeting of the Univer- Clinics, Lund, Sweden, September 11, 1952.

Fig. 2. The needle is introduced through the suprasternal notch into the aortic arch.

TECHNIQUE

The exact position of the aortic arch in frontal and lateral views is determined through fluoroscopic screening by the operator, immediately before the punchure in each case.

With the patient in a supine position and his face turned to the left, the needle is introduced into the anterior mediastium (Fig. 2). Local anaesthesia is used. The needle is directed in its course according to the findings at the fluoroscopy. The vigorous pulsations of the aortic arch are left with the needle, usually at a depth of 4 to 7 cm. The skin around the needle can be elevated in a tent-like manner before the wall of the arch has been perforated. When the tip of the needle has been inserted through the aortic wall, the vigorous pulsations are no longer felt, and the skin cannot be elevated aronud the needle. Continuous infusion is maintained through the needle when recordings are not being taken.

Premedication is not necessary. If pr local anaesthesia has been administrated, patient experiences little or no discomf Complications have not been encountere

PRELIMINARY RESULTS

Our material is so far limited, bu broader use of the method is planned variety of cardiovascular lesions. The hod is evidently a safe and simple wa obtaining accurate recordings of the pres pulses in the aorta.

The aortic pressure curve from a cas patent ductus arteriosus, verified at op tion, is shown in Figures 3.

SUMMARY

A new method for recording aortic p sure pulses is described. The aortic arc punctured percutaneously from the r and recordings are made through the ne

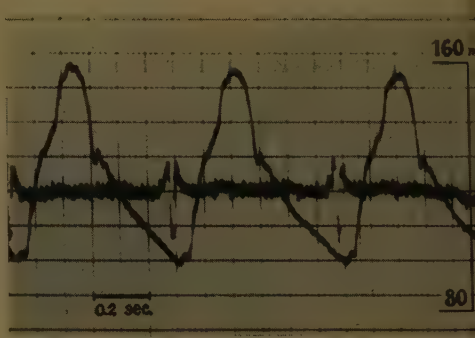

Fig. 3. Aortic pressure curve from a case of p ductus arteriosus, woman aged 20 years. "nipple-like" type of curve. At operation ductus was hour-glass-shaped, and its smalles cumference was 22 mm. There was no pulm hypertension.

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A SIMPLE METHOD FOR THE DETERMINATION OF FUNCTIONAL RESIDUAL AIR VOLUME

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(Received for publication October 22, 1952)

The methods most commonly used for the determination of RV are described by Comroe (1950). A "constant volume method" with helium as indifferent gas — readily determined in a catharometer — is supposed to be the most accurate and quickest.

The method described in the following requires a minimum of equipment and has been found to be accurate and easy.

PRINCIPLE

The concentration of N_2 in the lungs is lowered by breathing pure oxygen. The subject then hyperventilates til complete mixing in an air-filled (high N_2 percentage) sys of known volume and FRV is calculate the usual way from the N_2 per cent in final mixture.

EQUIPMENT

Mouthpiece or naso-oral mask, a stand spirometer with expiratory and inspirat valves, a rubber bag, a three-way stop-c a nitrogen analyzer and an oxygen cylin

PROCEDURE

The subject, who has to be in a res state, is well instructed about the necessit his cooperation. Equipped with a noseclip a mouthpiece he takes 5—6 deep and s inspirations from an oxygen-filled sp meter (sufficient oxygen beeing supp from an oxygen-cylinder) exhaling to The expiratory tube is then connected the spirometer and the oxygen supply f the cylinder stopped.

VC, CV and SV are then determi followed by a spirometer tracing of 8- minutes duration of which at least the 3—4 minutes have to be even and regu The N_2 per cent in the lung-spirom system is now between 3 and 6. At the of a normal expiration the subject is con ted with a rubber bag containing a kn volume of air (4—5 litres). He is instru to breath deeply and fast (using 3—4 conds per breath) for 20—25 seconds ar

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All volumes are corrected for temperature (to 37° C), vapor pressure (to saturation at 37° C) but not for barometric pressure.

The following abbreviations are used:

TC = Total capacity — the volume of air in the lungs after maximal inspiration.

VC = Vital capacity — the volume from maximal expiration to maximal inspiration.

CV = Complementary volume — from normal expiration to maximal inspiration.

SV = Supplementary volume — from normal expiration to maximal expiration.

RV = Residual volume — the amount of air remaining in the lungs after maximal expiration.

FRV = Functional residual volume — the amount of air remaining in the lungs after normal expiration (SV + RV).

N_2 = Nitrogen.

sp = spirometer.

alv = alveolar air.

n connected with the air outlet on the ɔcock. Samples from the spirometer and bag are anlyzed on N_2.

Jsually the procedure is repeated to get a ɔlicate determination.

The "Quick Analyzer" described by P. F. ɔolander (1942) has been used for deter-ıing the N_2 per cent. In order to avoid ıcturing the rubber tubing (which even-lly leads to leaks) and to avoid absorption d running into the syringe the analyzer been slightly modified as shown in . 1. The absorption fluid through which air samle is introduced is pyrogallic acid KOH[1], so that both O_2 and CO_2 are orbed and only N_2 remains.

DISCUSSION

The main sources of errors in the deter-ıation of functional residual air are:

) Unequal distribution of the "indica-tor" gas in the lungs.

) Oxygen storage effect.

) Changes in the volume of "indicator gas" during the test period due to diffusion from the pulmonary capil-laries to the lung air or the reverse.

The sources of errors have previ-ously been thorougly discussed, espe-cially by Lassen et al. (1937), Cour-nand (1940/41), Gilson (1949), Rahn (1949) and Georg (1952) and will not be repeated here.

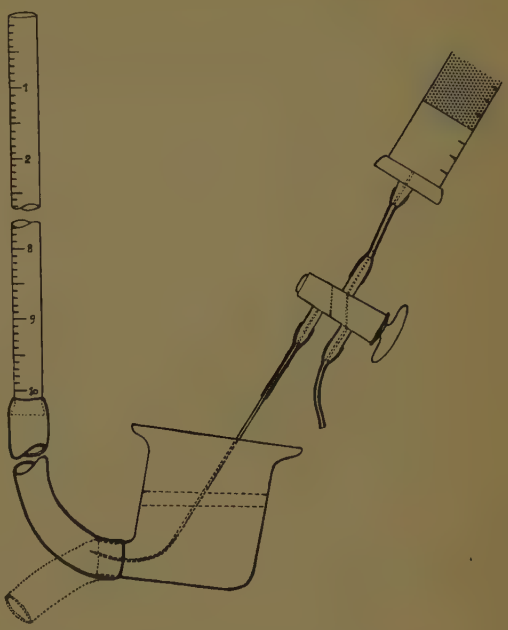


Fig. 1. Modification of "Quick Analyzer". The canula is oriented so that the gas can enter the glass tube.

After the introductory hyperventilation used in this method, the N_2 per cent in the lung-spirometer system vill be about 2—4 per cent (fig. 2, exp. 2). During the O_2-breathing it will rise slowly (0.1—0.2 per cent per minute).

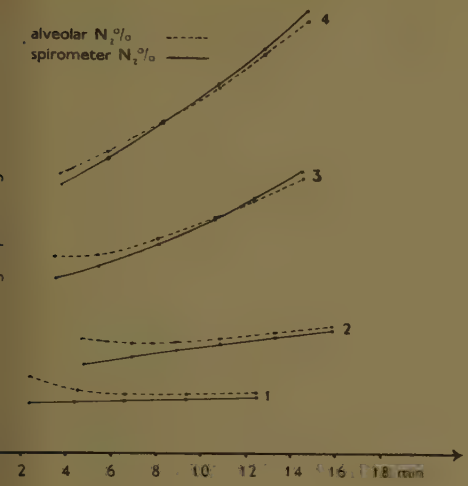
After 10 minutes there is less than 0.3 per cent difference between alv. N_2 per cent and sp. N_2 per cent. By determining sp. N_2 per cent instead of alv. N_2 per cent the resul-ting error in FRV is only 40 ml. An error of the same magnitude will result from absorp-tion of a small amount of nitrogen by the blood during the lung-bag breathing. These two errors influence the results in oposite directions. It seems justified under most conditions to disregard them.

One part (volume) pyrogallic acid (20 g pyro-c acid + 75 g aq.dest.) is mixed with 5—6 s of concentrated KOH (3 parts (weight) of H + 2 parts of water). When covered with cm layer of paraf. liq. in a dark bottle, this ɛent can be kept for a very long time. Better ed one day before use.

Table I. *Details of 33 Observations of One Normal Subject.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TC	6.20	6.18	6.31	6.30	5.90	6.10	6.00	6.20	6.20	6.00	6.10	5.95	5.95	6.20	6.17	6.17	6.05	6.10	5.95	6.15
VC	4.75	4.80	4.80	*4.95*	4.50	4.50	4.60	4.65	4.65	4.70	4.70	4.60	4.60	4 80	4.75	4.75	4.60	4 60	4.60	4.70
VC/TC %	76.5	77.7	76.0	78.5	76.2	73.9	76.7	75.0	75.0	78.3	77.0	77.3	77.3	77.4	77.0	77.0	76.0	75.4	77.3	76.4
CV	2.85	2.80	2.81	3.05	2.60	2.70	2.75	2.80	2.80	2.80	2.80	2.60	2.60	2.80	2.75	2.75	2.60	2.60	2.57	2.65
CV/TC %	46.0	45.3	44.4	47.8	44.0	44.1	45.9	45.2	45.2	46.7	45.9	43.7	43.7	45.2	44.6	44.6	43.0	42.6	43.2	43.1
SV	1.90	2.00	2.00	1.90	1.90	1.80	1.85	1.85	1.85	1.87	1.90	1.95	1.95	2.00	2.00	2.00	2.00	2.00	2.05	2.10
SV/TV %	30.6	32.4	31.6	30.1	32.2	29.5	30.9	29.8	29.8	31.2	31.2	32.8	32.8	32.2	32.4	32.4	33.0	32.8	34.4	34.1
FRV	3.37	3.37	3.50	3.25	3.30	3.40	3.30	3.40	3.40	3.25	3.32	3.32	3.32	3.45	3.45	3.45	3.45	3.46	3.40	3.50
FRV/TC %	54.5	54.4	55.4	*51.5*	55.9	55.7	55.0	54.9	54.9	54.3	54.4	55.7	55.7	55.6	55.9	56.0	57.0	56.7	57.1	56.9
RV	1.47	1.37	1.50	1.30	1.37	1.60	1.42	1.50	1.50	1.35	1.40	1.40	1.30	1.40	1.40	1.40	1.45	1.47	1.35	1.40
RV/TC %	23.7	22.2	23.8	20.6	23.2	26.2	23.6	24.2	24.2	22.5	22.9	23.5	22.7	22.6	25.6	25.6	24.0	24.1	22.7	22.8

	21	22	23	24	25	26	27	28	29	30	31	32	33	Mean	S. D.	Var. Coeff.	Range
TC	*5.65*	6.15	6.40	6.45	6.45	6.45	6.45	5.95	6.20	5.75	6.15	6.25	*6.60*	6 16	0.208	3.37 %	5.65 — 6.60
VC	*4.50*	4.65	4.70	4.70	4.85	4.85	4.85	4.65	4.65	4.50	4.65	4.75	4.85	4.69	0.113	2.42 %	4.50 — 4.95
VC/TC %	*79.6*	75.6	73.4	*72.8*	75.2	75.2	75.2	78.1	75.0	79 0	75.6	74.6	73.5	76.2	1.407	1.84 %	72.8 — 79.6
CV	2.40	2.75	2.90	2.90	*3.10*	3.10	3 10	2.60	2.60	*2.40*	2.75	2.90	3.10	2.77	0.184	6.63 %	2.40 — 3.10
CV/TC %	42.5	44.7	45.3	45.0	48.1	48.1	*48.1*	43.7	*41.9*	4.21	44.7	45.6	47.0	44.8	1.71	3.81 %	41.9 — 48.1
SV	2.05	1.90	1.80	1.80	1.75	*1.74*	1.75	2.10	2.10	*2.10*	1.90	1.85	1.75	1.92	0.110	5.70 %	1.74 — 2.10
SV/TC %	36.3	30.9	28.1	27.9	27.1	27.1	27.1	35.3	33.8	*36.8*	30.9	29.1	*26.5*	31.3	2.670	8.53 %	26 5 — 36.8
FRV	*3.20*	3.40	3.45	3.50	3.40	3.25	3.40	3.35	*3.60*	3.35	3.45	3.45	3.50	3.39	0.088	2.58 %	3.20 — 3.60
FRV/TC %	56.6	55.3	53 9	54.3	52.7	50.4	52.7	56.2	58.0	*58.7*	56.0	54.3	53.0	55.1	2.710	4.91 %	51.5 — 58.7
RV	*1.15*	1.50	1.65	1.70	1.60	1.50	1.60	1.30	1.50	1.25	1.50	1.55	*1.75*	1.45	0.127	8.77 %	1.15 — 1.75
RV/TC %	*20.4*	24.4	25.8	26.4	28.0	23.3	*28.0*	21.8	24.2	21.9	26.3	24.4	26.5	24.0	1.88	7.83 %	20.4 — 28.0



2. Illustrating how the N_2 percentage in the olar gas and in the spirometer gas is altered ng breathing in a spirometer with varying me and varying amounts of N_2 in the lung-ometer system. In Exp. 1 the volume was t 110 l, in Exps. 2, 3 and 4 it was 15 l. In s. 1 and 2 the N_2 per cent was low after the rimental person had taken 5—6 deep breaths the O_2-filled spirometer. In Exps. 3 and 4 nitial removal of N_2 was less complete. Exp. 2 esponds to our standard conditions with 3—5 cent N_2 in the lung-spirometer system. The amount of oxygen is so large that the N_2 cent increases slowly. This gives a small ibution error and the oxygen storage effect for part of the method is also slight. Exps. 3 and ve large distribution errors. Exp. 1 requires extra technical equipment.

spirometer N_2 per cent ————————
alveolar N_2 per cent — — — — — —

uring the lung-bag breathing, the volu-ric change from CO_2 output and O_2 up-is negligible.

he method has the advantages of being N-uptake" method and of using an indicator gas with great difference in percentage between the lungs and the measured volume. The main errors, namely from unequal distribution of the indicator gas and from diffusion of N into the blood, are small even with the short mixing period. If desired, the method can easily be converted to a "constant volume method".

Table I shows the results of 33 repeated determinations on one normal subject in the course of 6 months.

In Table II these results are compared with 27 determinations on one normal individual by Whitfield (1950) using the helium method.

Duplicate determinations of FRV were done on 61 enlisted young men without any previous experience in such tests. The diffe-

Table II. *Results of 33 repeated determinations of different lung volumes using the present method on one normal subject (♂, 43 years), in the course of 6 mounths, compared with results of 27 separated deter minations by Whitfield on another normal individual using the helium-method.*

	33 determinations			Whitfield — 27 determinations		
	M	SD	Var. coeff.	M	SD	Var. coeff.
TC	6.16 l	0.208	3.37	6.346	0.238	3.9
VC	4.69 l	0.113	2.42	4.514	0.110	2.4
VC/TC % ...	76.2 %	1.407	1.84	71.333	2.511	3.52
CV	2.77 l	0.184	6.63	2.96	0.125	4.2
CV/TC % ...	44.8 %	1.71	3.81	46.296	2.504	5.41
SV.....	1.92 l	0.11	5.70	1.546	0.135	8.6
SV/TC %....	31.3 %	2.67	8.53	25.037	2.252	8.99
FRV	3.39 l	0.088	2.58	3.396	0.253	7.4
FRV/TC % ..	55.1 %	2.71	4.91	53.704	2.502	4.66
RV	1.45 l	0.127	8.77	1.83	0.206	11.3
RV/TC % ...	24.0 %	1.88	7.83	28.667	2.507	8.74

rence between duplicates was within the range 0—550 ml with a standard deviation of 159 ml.

The method only been applied to normal subjects. It is possible that alveolar air samples for N_2 per cent determinations are necessary on patients with pathological lung changes like emphysema which impair the mixing conditions.

SUMMARY

A "closed circuit, nitrogen-uptake" method for determination of FRV, requiring a minimum of equipment and analytical work is described. The main sources of errors are the same as for similar methods. Results from normal subjects compare favorably with the helium method.

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THE TOXICITY OF SYNTHETIC HEPARIN SUBSTITUTES

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(Received for publication October 25, 1952)

Since Bergström (1935, 1936) discovered anticoagulant effect of synthetic polysaccride polysulphuric acid esters, there has n a constant search for an inexpensive thetic substitute for heparin sufficiently e to be used in human therapy.

During our first investigations, we entered difficulties in estimating the toxi- of such substances, (Astrup, Galsmar & lkert, 1944) but after it was found by er (1945 a) that they produce agglutina- of the blood platelets, more detailed rmacological studies of these substances re possible, (Piper 1945 b, 1946 a, b, 7). Some of the synthetic substances even cipitated fibrinogen in the blood, (Astrup Piper 1946, Barsøe & Selsø 1946).

These experiments led to the conclusion t platelet agglutination and precipitation fibrinogen are toxic manifestations. ing these criteria it is therefore possible obtain information about the toxicity of se polysaccharide derivatives, which only asionally cause immediate death when inted into animals. We were not successful finding synthetic substances which could nd these tests (Astrup & Piper 1945 a, b; unpublished).

Recently a number of new derivatives have eared, and some of these have even been d as anticoagulants in human therapy.

We have studied some of these new substances. The results are presented here together with some observations on the method of estimating the toxicity, which are important if reliable results are to be obtained.

MATERIALS AND METHODS

Hyaluronic acid sulphuric acid ester: This derivative was described by Balazs, Högberg & Laurent (1951) and Pantlitsehko, Schmid, Seelich & Kaiser (1951) and was kindly placed at our disposal by Dr. Högberg, Leo Ltd., Hälsingborg, Sweden.

Xylan sulphuric acid ester: This substance was prepared by Husemann, v. Kaulla & Kappesser (1946) and is put on the market under the trade name *"Thrombocid"*. Samples were kindly received from Dr. v. Kaulla and from C. F. Boehringer & Soehne, Mannheim, Germany.

Alginic acid polysulphuric acid ester: This ester was prepared by and kindly obtained from Wyeth, Inc., Philadelphia, U. S. A. It has been studied by Sorenson & Wright (1949) under the trade name *"Paritol"*. (Sample marked "Paritol C", lot No. 69201).

Cellulose trisulphuric acid and chitin disulphuric acid were prepared as described by Astrup, Galsmar & Volkert, 1944. Heparin was obtained from Løvens kemiske Fabrik, Copenhagen.

Blood samples were centrifuged for 30 sec. at slow speed (about 1000 revolutions per min.) or left standing for one hour for natural sedimentation. Samples were then removed by a glass capillary. Platelets were counted in 16 squares of a Thoma counting chamber (0.05 mm deep). Results are given in thousands per cubic mm of the final

plasma mixture. The counts were made by normal microscopy or preferably by phase contrast microscopy where the platelets are very easily recognized as particles with a clear bluish tint.

EXPERIMENTAL

The method preferred by Piper (1945 a, b) for estimating in vitro the toxicity of synthetic anticoagulants of the heparin type consists in bleeding a rabbit from the carotid artery directly into test tubes containing the solution under investigation. In some cases citrate was added to the solution, and this addition did not interfere with the results.

Piper also drew blood from an ear vein of a rabbit through a paraffined cannula into a paraffined syringe (2 ml) containing a citrate solution of the anticoagulant. The final mixture consisted of 0.6 ml 3.5 per cent citrate containing the anticoagulant and 1.4 ml blood. With this procedure he obtained results indentical with those obtained by bleeding directly from the carotid artery into the test solutions without citrate.

In the experiments to be described first, *rabbit blood* was obtained in $^1/_{10}$ volume of 3.5 per cent citrate by heart puncture using a paraffined syringe or a syringe containing olive oil. The citrated blood was poured into a silicone treated beaker and aliquots were then added to a solution of the anticoagulant in a paraffined or silicone treated tube containing an excess of citrate. The following is an example of an experiment performed in this manner:

To tubes containing 0.5 ml. 3.5 per cent citrate and 0.5 ml of a 0.5 per cent neutral solution of the anticoagulant in physiological NaCl were added 2.5 ml citrated rabbit blood obtained as described above. The free platelets were counted as described and gave t following results:

Control with 3.5 % citrate 3
Heparin, 0.5 % 2
Cellulose trisulphuric acid, 0.5 % ....
Chitin disulphuric acid, 0.5 % .....
Hyaluronic acid sulphuric acid, 0.5 %
Xylan sulphuric acid, 0.5 % .....
Alginic acid polysulphuric acid, 0.5 %

The agglutinated samples all contain few free platelets but had a certain numb of smaller or larger agglomerates of pla lets. The cellulose trisulphuric acid and c tin disulphuric acid were included becau these are known to have an agglutinati effect. It is seen that the new substances produce an agglutination, while heparin without effect.

Using *human blood* Piper (1945 a, b) a owed blood from an arm vein to run direc into tubes containing the solutions in a ma ner similar to that used in experiments w blood from the carotid artery of a rabb He obtained the same results with hum blood as with rabbit blood. Proceeding this manner we allowed 4.5 ml blood fr an arm vein to run directly into 0.5 ml the saline solution of the anticoagulant co tained in a silicone treated tube.

The following results are representati for such an experiment:

Control with 3.5 % citrat: 3
Heparin, 0.5 % 3
Cellulose trisulphuric acid, 0.5 % ....
Chitin disulphuric acid, 0.5 % .....
Hyaluronic acid sulphuric acid, 0.5 %
Xylan sulphuric acid, 0.5 % .....
Alginic acid polysulphuric acid, 0.5 %

These results compare with those obtained th rabbit blood.

We tried also to perform the test on citra- l human blood drawn with a syringe from arm vein. Unexpectedly with this proce- re we obtained results differing from ose described above. The blood was drawn o 1/10 vol. of citrate contained in a paraf- ied or oiled syringe and was poured into a icone treated beaker. Then 2.5 ml of blood s added to tubes containing 0.5 ml 3.5 per nt citrate solution and 0.5 ml of a neutral ution of the anticoagulant in saline:

The following is a representative experi- ent:

ntrol, 3.5 % citrate	205
eparin, 0.5 %	215
llulose trisulphuric acid, 0.5 %	130
itin disulphuric acid, 0.5 %	120
aluronic acid sulphuric acid, 0.5 %	135
lan sulphuric acid, 0.5 %	140
ginic acid polysulphuric acid, 0.5 %	150

The fall in platelet number is much smaller th this procedure than in the previous ex- ples. In single cases only a very slight l was observed. The human platelets are obably stabilized to a certain extent when ey are kept for some time in the presence citrate. This means that toxicity estima- ns performed on human blood obtained as re described do not give the true picture of toxicity of the substances tested. This ult is significant because this is the rou- e method for obtaining human blood.

The addition alone of citrate is not the se of this discrepancy. Experiments in ich citrate was added to solutions of the icoagulants in test tubes, and blood was d directly from an arm vein into the tube, gave agglutination for all the synthetic substances, just as in similar experiments performed without addition of citrate. The following experiment was performed with silicone treated tubes each containing 0.5 ml of the anticoagulant solution and 1.5 ml citrate. Then 4.5 ml human blood was bled directly from the arm vein into each tube:

The results were as follows:

Control with 3.5 % citrate	315
Heparin, 0.5 %	290
Cellulose trisulphuric acid, 0.5 %	25
Chitin disulphuric acid, 0.5 %	30
Hyaluronic acid sulphuric acid, 0.5 %	35
Xylan sulphuric acid, 0.5 %	60
Alginic acid polysulphuric acid, 0.5 %	55

The results of this experiment are in accordance with those obtained with rabbit blood. Other experiments, in which the blood was allowed to run into pure citrate solution before it was added to the anticoagulant solutions, indicate that the stabilizing effect of citrate on human platelets may need time to develop.

DISCUSSION

The results here presented show that the methods used for toxicity estimations with rabbit blood cannot be applied unmodified on human blood, but that certain precautions are necessary in order to obtain true values for the toxicity.

When reliable methods are used, all the synthetic substances studied here appear toxic. This is of considerable significance because all the new substances here investigated have been tried on human patients. Occasionally single cases gave results deviating from those reported here. With one exception these occurred too irregularly to

interfere with the main results. However, in the case of xylan sulphuric acid a number of human blood samples produced no agglutination, though all samples of rabbit blood did agglutinate.

The sulphuric acid ester of hyaluronic acid was studied by Schmid (1951) and tried on patients. He suggests that this acid is identical with heparin, but our comparative toxicity determinations stress the results of Balazs, Högberg & Laurent (1951) concerning the non-identity of heparin and the hyaluronic acid sulphuric acid ester.

The properties of xylan sulphuric acid ("Thrombocid") were described by Husemann, v. Kaulla & Kapesser (1946) and v. Kaulla & Husemann (1946), but no platelet agglutination tests have been published for this substance. This substance has been tried in the clinic by Halse (1949, 1950), Halse & Schmitz (1949) and Schulze (1950). The difference in the results obtained with this acid on different blood samples probably indicates a variation in the sensitivity of the platelets, and a relatively low toxicity of the xylan compound.

The properties of the alginic acid polysulphuric acid ("Paritol") were described by Seifter & Begany (1948), and the substance has been investigated in the clinic by Sorenson, Seifter & Wright (1948) and Sorenson & Wright (1949, 1950 a, b). Alginic acid sulphuric acid ester was also prepared and investigated in vivo in rabbits by Meunier, Molho, Moraux & Cotte (1950), who observed its ability to precipitate fibrinogen.

In the discussion of the safe applicability of "Paritol" the question of the platelet agglutination was considered, but in human beings no effect on the platelet count w found by these authors. This result does agree with our experiments, wich are ba on a different method. In our opinion so of the toxic manifestations seen in so cases after the administration of "Parit may be due to the formation of emboli c sing infarctation especially in the lungs. S cases with no gross pathological manife tion of embolism have been produced exp mentally in rabbits by Piper (1945 b, 1 b). The existence of these conditions revealed only after autopsy. *It is impossi to draw conclusions about the action of th substances from the observation of clini cases, because any clinical signs of emboli will be interpreted as a manifestation of patient's original thrombo-embolic conditi and not as a toxic effect of the anticoagula*

So far we have found no synthetic hepa substitutes which do not produce plate agglutination under the experimental c ditions here described. It has been a mat of dispute how the molecular size of anticoagulant influences the toxicity, an number of substances with rather low mo cular weight have been triel. Thus substan prepared from dextran, inulin and a numb of other polysaccharides have been investi ted (Grönwall, Ingelman & Mosiman 1 and Ingelman 1946). These authors a prepared dextran sulphuric acid esters w varying molecular weight, but found th all to be toxic. Very recently, however number of dextran sulphates have been p pared by Ricketts (1951, 1952). Some these did not precipitate fibrinogen and p duced no agglutination of the platelets. Th may be sufficiently non-toxic to warran trial in human therapy (Walton 1951, 195

t may be added that although heparin in ge doses prevents the agglutination of telets as observed by Solandt & Best 940) and Baronofsky & Quick (1943) aller doses may produce an agglutination the platelets (Copley & Robb 1942 a, b), llar & Jaques 1948, Quick, Shanberge & fanini 1948). This effect is however st pronounced in dogs; it is probably ibited by citrate (Copley & Robb 1942 a). our experiments with rabbit and human od we have always found that the sample taining heparin agrees with the control taining citrate. This result compares with observations of Quick, Shanberge & Stefini (1948).

SUMMARY

1. The platelet agglutinating effect of the lowing three new heparin substitutes was died: a hyaluronic acid sulphuric acid er, a xylan sulphuric acid ester (Thromcid) and an alginic acid sulphuric acid er (Paritol). They were all found to glutinate the platelets and are too toxic for nical use.

2. The agglutination test is discussed in light of some discrepancies found between behaviour of rabbit blood and human od. Citrate appears to stabilize the human telets.

This investigation was aided by a grant m the Josiah Macy, Jr. Foundation, New rk. Dr. Erik Henrichsen kindly controlled ne of our platelet counts.

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THE SIGNIFICANCE OF AUTO-AGGLUTININS

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It is believed that auto-agglutinins, also led non-specific cold agglutinins, can be rmed in at least two ways. They may be oduced by auto- immunisation or they may of heterogenetic bacterial origin (Wiener, 51). Immunisation to one's own red corscles may result, in some ill-understood y, from corpuscular destruction, and autoglutinins are commonly associated with quired hemolytic anemias. The cold agglutins of virus pneumonia probably belong to e same category; the virus may alter the l receptors in such a way that auto-antidy formation is evoked.

A moment's reflection should make it evint that there would be a fundamental difrence between the two types when the antidies are well developed. Agglutinins resulg from auto-immunisation would be excted to act upon the individual's own cells the same or almost the same extent (titre d thermal amplitude) as other cells conning the same antigens. Those of heteronetic bacterial origin would be expected to upon the individual's cells only in the d because such agglutinins, active at higr temperatures, would be neutralised as ickly as they are produced, while those ing at lower temperatures would survive. n the other hand antibodies to antigens not present in the individual's red cells, active also at higher temperatures, could develop without hindrance. The heterogenetic type would be expected to be present in most sera.

It is felt that it would be useful to abandon, from the clinical if not from the serological point of view, the classification of cold agglutinins into non-specific and specific types, in favor of one which distinguishes cold agglutinins arising from auto-immunisation from those solely of heterogenetic origin. The former sub-division is probably not valid because it is suspected that non-specific agglutinins may consist of multiple specific components (Bird, 1951), which may exhibit agglutinin linkage (Bird, 1952).

It is also suggested that diagnostic cold agglutinin tests might be of much more value if they are carried out in the cold, at room and body temperature, not only against 0 cells, which is the usual practice, but also against the patient's own cells. In some cases this might help to exclude cold agglutinins of heterogenetic origin which are usually of no diagnostic significance. This would be particularly important in the tropics, where the generally greater sub-clinical bacterial assault gives rise to fairly high titres of heterogenetic cold agglutinins in normal persons. In our laboratories we use 0 cells, 'compatible' A or B cells, and the patient's own cells, for every

Table I.

Serum No.	Clinical Diagnosis	Group	Titre at 4°—6° C against various cells				Probable origin
			O	A_1	B	Own	
1	Virus pneumonia	O	64 (6)			256 (6)	Auto-immunisatio
2	Virus pneumonia	B	256 (6)		256 (6)	256 (6)	Auto-immunisatio
3	Polycystic lung	O	128 (6)			16 (6)	Heterogenetic.
4	Anemia due to bleeding piles	O	32 (30)			8 (6)	Heterogenetic.
5	Healthy blood donor[1].	O	128 (30)			16 (6)	Heterogenetic.
6	Acquired idiopathic hemolytic anemia	O	128 (37)			64 (30)	Auto-immunisatio
7 (a)	Acquired idiopathic hemolytic anemia	A_1 B	128 (37)	128 (37)	16 (30)	8 (30)	?Heterogenetic (a antibodies, not developed).
7 (b)	Old case of acquired idiopathic hemolytic anemia.....	A_1 B	128 (37)	128 (37)	64 (37)	64 (37)	Auto-immunisatio (same case as 7
8	Tropical eosinophilia ..	B	128 (37)		128 (37)	128 (37)	Auto-immunisatio
9	Macrocytic anemia of pregnancy	A_1	32 (37)	32 (37)		32 (30)	?Auto-immunisati
10	Healthy blood donor...	A_2 B	256 (6)	256 (30)	256 (6)	256 (6)	The anti-A_1 agg nin active at r temperature is doubtedly het genetic.

The figures in parentheses indicate whether the antibodies acted in the cold only (6° C) or room (30° C) or body temperature (37° C).

test. A rising titre against the patients own cells is considered to be of the greatest diagnostic significance in cases of Virus pneumonia.

Figures which support this opinion are given in the accompanying table. The data has been taken from our records and is therefore highly selected material. It is not suggested that all sera will give a clear in cation of the origin of the auto-agglutini In fact a great many will not do so especia if the agglutinins are active only in the co

It is evident that if the donor of serum is suspected of having Virus pneumonia cold agglutinin titre against 0 cells, if con dered alone, might prove misleading.

It is realised that specially absorbed a globulin sera (Crawford & Mollison, 19 might be able to distinguish the two ty of auto-agglutinins but it is considered t

[1] The expression healthy blood donor indicates that he was in good health at the time of bleeding, gave no history of having had serious illness, transfusion or injection of blood.

will be quite some time before such sera ecome generally available.

One of the cases included in the table lls for some comment, Sera 7 (a) and 7) are from the same patient, the latter mple having been taken six months after e former. At this time the patient was no nger anemic. The auto-antibodies appear be the effect rather than the cause of her iemia. Agglutination *in vitro* at 37° C does ot necessarily mean that agglutination will ke place *in vivo* (see also serum 8). If chnical fallacies have been excluded, some otective mechanism must exist. The nature this protective device, if any, might use- lly be investigated.

Sera 4 and 5 may be examples of sera cently described by Bhende et al. (1952), of anti — M, anti — P, or any other rare o-agglutinins of spontaneous origin'.

SUMMARY

It may be useful to distinguish cold agglutinins arising from auto-immunisation from those of heterogenetic bacterial origin. To this end a modification in diagnostic cold agglutinin tests is suggested.

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ATTEMPTS TO PRODUCE EXPERIMENTAL LIPID NEPHROS WITH TRIDIONE

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Repeated reports of lipid nephrosis following the treatment of epilepsy with tridione — 3,5,5-trimetyloxazolidin-2,4-dion — (Barnett, Simons 1948, White 1949) provided the basis for the present investigation. This occasional complication after tridione therapy is particularly interesting, because the typical nephrotic syndrome which develops is reversible as shown by fact that it disappears on cessation of the tridione administration and may even re-appear on recommencing the treatment.

As far as we know the classical picture of *pure* lipid nephrosis has not been induced experimentally up to the present, and it therefore seemed interesting to find out whether the administration of tridione to experimental animals could bring about the same condition. The experimental induction of lipid nephrosis would appear to be of some help in throwing light on the obscure pathogenesis of the nephrotic syndrome.

METHODS

Experimental animals. The animals used were:

1. Female rats from a single inbred strain, weighing 150—200 g.
2. Rabbits of a mixed experimental breed, weighing 2—4 kg.
3. Guinea pigs of a mixed laboratory strain, weighing 400—600 g.

Tridione administration. The commercially av able tridione preparation supplied by the Ab Laboratories was used for the experime Attempts to dissolve the tridione in an acid alkaline solution failed and it was there suspended in water and administered via a ston tube. With the doses given the animals bec intoxicated, and died sooner or later. In t experiments the animals were killed before intoxication became marked.

The experiments were started in the autum 1949. One year later the commercially avail preparation was noticed to have changed character. It had become more coarsely gran and hygroscopic. At the same time the effec the drug was found to have changed. This wil discussed later.

Lipid Determination Methods.

The determinations were made on serum. Ext tions of the lipids were carried out by the Egsg method 1948. After careful evaporation of extraction medium, the lipids were redissolve chloroform. From several portions of this chl form, determinations were made of:

Total lipids, by means of evaporation and we ing of the residue.

Cholesterol, according to the method of The and Widström 1931 with the Liebermann-Burc color reaction.

Lipid phosphorus, after evaporation of the chl form, digestion of the residue with sulphuric redissolving in water, and phosphate determina according to the method of Briggs 1922. *Determ tion of the tributyrinase and phosphomonoeste activity* in tissue suspensions of liver and kidne well as in serum was made according to the t nique earlier described by Svanborg (1951).

Table I.

bbits No.	Duration of tridione therapy in months	Mean dose in mg/day	Total lipids mg/100 ml			Cholesterol mg/100 ml			Lipid P mg/100 ml			Proteinuria
			Before administration	Highest values	Percentage increase	Before administration	Highest values	Percentage increase	Before administration	Highest values	Percentage increase	
3	2.5	1100	120	444	270	12?	21?	(75)	1.6	2.7	69	o
4	1.0	900	330	2040	518	53	258	387	6.3	12.6	100	+
5	11.0	900	160	1660	938	23	75	226	1.7	9.7	471	o
6	5.5	900	350	1820	420	26	125	381	3.7	4.5	22	+
7	16.0	900	140	900	543	23	101	339	1.8	5.9	228	o
8	4.5	1500	-	-	-	20	44	120	-	-	-	+
9	6.0	900	360	840	133	20	69	245	1.9	8.1	325	+
12	15.0	900	200	950	375	20	40	100	1.3	6.5	400	o
21	4.0	600	-	-	-	39	114	192	3.7	7.0	89	+
23	3.0	600	-	-	-	44	88	100	-	-	-	+
00	11.0	900	200	1540	670	25	70	180	1.2	6.4	433	+
01	9.5	950	290	700	141	38	61	61	1.9	4.5	137	o

RESULTS

A. *Oral administration of tridione to bbits.* Table I.

The experiments show that there develops rabbits a *hyperlipemia,* which is appreciy marked. The raised level of the blood ids can be compared in magnitude with the perlipemia following either bilateral nephctomy or ligation of the ureters. The pertage increase of the total lipid concentran exceeds on the average that of cholesol and phospholipids. This seems to signithat a rise in neutral fat makes an impor- contribution to the lipid increase.

The total *plasma proteins* showed a slight *rease,* which was due chiefly to a rise in mma globulin.

In more than half of the experimental mals (7/12) a mild *proteinuria* was enntered. It was, as a rule, less than 0.1 per t. The proteinuria bore no relationship the development or intensity of the hyperlipemia and was detected chiefly towards the terminal stages. In the urinary sediment, scattered hyaline and squamous casts were found along with a few white cells, but no red blood corpuscles.

At the peak of the hyperlipemia the *kidneys* were shown to be the site of marked *fatty deposits.* These were not doubly refractile and were located, in particular, in the proximal tubules and identifiable by their staining with Sudan III. Furthermore, degenerative changes of the nephrosis type could be seen. Liver slices taken at the same time showed no definite pathological changes, apart from, in some cases, small fatty deposits in the peripheral parts of the lobules.

As regards the enzyme activity in tissue suspension there were presumable no demonstrable changes in the phosphomonoesterase activity. On the other hand, the tributyrianse activity in the kidney suspension was seen

to have fallen slightly below the normal levels. As mentioned earlier, it was observed in the autumn of 1950 that the commercial tridione preparation had changed in character, the granules becoming coarser and more hygroscopic. At the same time, neither the earlier demonstrable effect on the blood lipids, nor the renal fatty changes could be reproduced. The animals no longer showed signs of intoxication. In view of the small number of determinations made on enzyme activity prior to the change in tridione, it was not possible to draw any definite conclusions regarding the change in the enzyme activity.

B. *Oral administration of tridione to rats.* Table II. The duration of the treatment extended over 3 to 10 days.

Table II.

Rat No.	Duration of tridione therapy in days	Total tridione administration in mg	Fatty deposition observed microscopically	
			Kidney	Liver
24	3	900	+ + +	+
25	4	1050	+ + +	+
26	5	1650	+ +	o
27	5	1650	+ +	+
28	2	600	+ + +	o
29	2	600	+ + +	o
G 4	10	1900	+ + +	o
G 10	5	1400	+ + +	o
G 11	6	1800	+ + +	o
G 12	6	1800	+ + +	o
G 13	6	1800	+ +	o
G 13	6	1800	+	o
St 1	59	5100	+ + +	+
St 3	2	750	+ + +	o
St 4	2	750	+ + +	o

The experiments show that, after giving the tridione, *a fatty change is detected in kidney sections* stained with Sudan III and this accumulation of fat was mostly confi to the proximal tubules. Control experime in animals not given tridione, did not sh similar changes.

C. A. smaller series of similar expe ments in *guinea-pigs* (5 animals) likew resulted in *pronounced renal fatty chang* Two of the animals developed a moder proteinuria. Table III.

Table III.

Guineapig No.	Duration of tridione therapy in days	Total tridione administration in mg	Fatty deposition observed microscopically	
			Kidney	Liver
1	43	9900	+ + +	+ +
2	30	7200	+ + +	(+)
3	30	9600	+ + +	(+)
4	16	5200	+ + +	+ +
5	2	900	+ + +	+

COMMENTS

Sometimes a fall in weight occurred dur the tridione administration. Therefore possibility that the hyperlipemia may be simply to a fat mobilization similar to t met with in starvation must be consider Our own studies on starving rabbits sh in fact, a rise in blood phosphatides cholesterol equal in magnitude to that fou in tridione-fed animals, but we did not f the *total* lipid levels of the latter. Howev even after prolonged starvation, neither r nor rabbits revealed any tubular fatty ch ges, nor was there any proteinuria. Mo over, the hyperlipemia developed in tridio fed animals even when they had a good ap tite and were gaining weight. In a series rat experiments the combination of gluc (4 gm. daily) with tridione feeding had

eventive effect on the fatty changes in the ney. Thus a hunger hyperlipemia would m to be excluded from the list of possible sal factors. Control studies in rabbits ewise showed that the loss of blood due to taking of numerous samples could be minated as a possible cause of hyperlipe- a (Boggs & Morris 1909). During the rse of each experiment the non-protein rogen did not rise except in the terminal ases.

Since the prolonged administration of ious amounts of tridione did not lead to development of the true nephrotic syn- me, various attempts were made to pro- ce renal damage in tridione-fed animals,

Production of allergic nephritis of the type described by Ahlström (1936). and produced by immunization with horse serum + staphylococcal toxins.

Infection with Staphylococcus aureus (subcutaneous abscesses).

Administration of toxins (subcutaneous streptococcal and intravenous staphylococcal toxins).

These experiments produced neither a rked increase in the proteinuria, nor hypo- teinemia, nor edema, nor a more pro- nced hyperlipemia.

We were therefore unable to reproduce true nephrotic syndrome occasionally t with as a complication during tridione rapy in humans. However, we have pro- ed renal changes of the nephrosis type ether with a distinct disturbance of the metabolism, by means of tridione. The erlipemia, one of the cardinal signs of d nephrosis, has thus been regularly induced as an isolated finding. All this speaks in favor of the conception that the lipemia of nephrosis is an independent entity in the nephrotic syndrome, and has no casual relationship to the proteinuria or the hypoproteinemia.

It must be emphasized that our results have been obtained with a particular commercial preparation of tridione and that other preparations have proved to be ineffective. We cannot, therefore, exclude the possibility that the experimentally induced changes are due to some unknown factor such as a solvent or some impurities incorporated in the tridione powder; however, it is more likely that there were differences in the solubility of earlier and later batches of the drug.

It seems unlikely that tridione in the therapeutic doses used in man could cause hyperlipemia. We have observed an epileptic who after 6 weeks medication (2 g daily) failed to reveal any significant blood cholesterol variations, and the electrophoretic pattern of his plasma did not alter.

SUMMARY

The oral administration of a tridione preparation has regularly produced pathological renal changes of a nephrotic types, with pronounced fatty degeneration in the proximal tubules in the rabbit, rat and guinea-pig, and marked hyperlipemia, involving neutral fat, cholesterol and phophatides, in the rabbit. In addition, about two thirds of the rabbits and guinea-pigs developed a slight proteinuria, which was not associated with hypoproteinemia or edema. Therefore all the typical signs of the nephrotic syndrome were not produced.

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MICROMETHOD FOR THE DETERMINATION OF CALCIUM AND MAGNESIUM IN SERUM

TITRATION WITH ETHYLENE-DIAMINE-TETRACETATE

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Schwarzenbach and co-workers (1946) scribed the complex-formation of metal ıs with certain compounds derived from ino-diacetic acid (Complexones) and used s complex-formation for titrimetric deter- nations of the respective metals. In parti- lar one of these compounds, ethylene-dia- ne-tetracetic acid (EDTA), was shown to suited for the titration of calcium and ıgnesium. The simplicity and usefulness this method was further increased through e discovery of suitable indicators. Thus ıurexide (ammonium purpurate) was own to be an indicator for the calcium ion chwarzenbach, Biedermann & Bangerter 46). The color-shift of this indicator wever (blue-violet to pink), is not a very arp one and the titrations tend to have fficult end points. A further difficulty is ıused by the instability of murexide in ıutions. Later Eriochrome Black T was scribed as indicator for calcium and mag- sium (Biedermann & Schwarzenbach 48). This dye is blue in alkaline solutions H 8—10) but wine-red in the presence calcium or magnesium ions. The end ints of titrations with this indicator will very sharp provided magnesium is pre- ıt in the solution.

Methods based on the use of EDTA and the indicators mentioned have been described for the determination of hardness of water (reviewed by Heald, Coates & Edwards 1950) and for the determination of calcium and magnesium in serum (Buckley, Gibson & Bortholotti 1951, and others). Compared to previous methods these procedures are simple and rapid. They are also readily applicable to small serum quantities (Sobel & Hanok 1951).

It follows that direct titration of serum with EDTA and Eriochrome Black T will determine the sum of magnesium and calcium present. The use of this indicator also for exact calcium determinations is to be preferred however, because of its better end point. Isolation of calcium by precipation as oxalate and subsequent titration then becomes necessary. In this situation some measure must be taken to keep calcium in solution in the presence of oxalate. In one method this was obtained by adding an excess of the magnesium complex of EDTA (Flaschka & Holasek 1951), calcium being preferentially complexed and held in solution. In another procedure described by Herman Nielsen (1952) calcium oxalate is dissolved in a known amount of EDTA, and the ex-

cess of EDTA determined by back-titration with a standardized solution of magnesium. A third possibility is decomposition of the oxalate, and this principle was adopted in the procedure to be described.

Obviously the determination of calcium after precipitation, combined with the direct titration of the same serum, will furnish the value for magnesium represented by the difference of the two titrations.

PRINCIPLE

The sum of calcium and magnesium is determined by direct titration. Calcium is determined after precipitation as calcium oxalate, the oxalate being decomposed with perchloric acid whereby calcium becomes directly titratable. Magnesium represents the difference between the values of direct titration and titration after precipitation.

REAGENTS

1. EDTA stock solution, 5 mEq/L.
   0.9302 g of di-sodium ethylene-diamine-tetracetate di-hydrate (Mol.w. 372.1) is dissolved in glass-distilled water and diluted to 1000 ml. Stable.
2. EDTA titrating solution, 0.25 mEq/L.
   5 ml of stock solution is diluted to 100 ml with water. Stable.
3. Ammonia-ammonium chloride buffer, pH 10.5.
   55 ml of concentrated ammonium hydroxide solution (sp.gr. 0.91) and 2.14 g NH_4Cl is dissolved and diluted to 500 ml with water.
4. Eriochrome Black T indicator solution.
   0.02 g of the dye is dissolved in 10 ml of 96 per cent ethyl alcohol. Stable for some weeks at room temperature, indefinitely at refrigerator temperature.
5. Standard calcium solution.
   0.2502 g calciumcarbonate is dissolved in a small volume of conc. hydrochloric acid and diluted to 1000 ml with water.
6. Magnesium complexonate solution.
   A solution of EDTA in ammonia-bu (approximately 0.1 per cent w/v) contai a few drops of indicator solution is titr to a stable purplish color with a tion of $MgCl_2 \cdot 6\,H_2O$ in water (approxima 0.1 per cent w/v). The mixture is carei titrated back to the blue end point with ED ammoniabuffer solution. Stable.
7. Concentrated perchloric acid, anal.grade.
8. Saturated solution of ammonium oxalate water.

PROCEDURE

1. Direct titration of calcium and mag sium in serum.

   0.05 ml of serum is pipetted into 0.5 of ammonia-buffer solution in a wi porcelain cup (50 mm in diameter) drop of indicator solution is added. mixture is titrated with EDTA (mEq/L) to a final greenish-blue co

   The low concentration of the titrat solution permits the use of a 2 ml bur graduated to 0.01 ml. It is necess to keep a sample which is titrated befo hand well beyond the end point for c parison.
2. Precipitation of calcium as oxalate subsequent titration.

   0.05 ml of serum is pipetted int small centrifuge tube (50×7 mm) v conical tip. 0.05 ml of ammonium oxa is added. 0.05 ml of distilled wate blown forcefully into each tube to ca mixing of the contents. The tubes left for precipitation for at least 6 ho after which they are centrifuged at 2 r. p. m. for 15 minutes. An exact p tion of the tubes during the centr gation is important, and may be ensu by fitting them into corks to be slip

into the centrifuge cups. The supernatant is carefully siphoned off. Approximately 0.01 ml of conc. perchlorid acid is added and the tubes kept in a boiling water bath for 30 min. The contents of each tube are quantitatively transferred to the titration cup by means of 3 portions of each 0.2 ml of the ammonia-buffer solution. 1 drop of indicator solution and 1 drop of magnesium complexonate solution are added and the titration carried out as under 1.

Standardization of titrating solution.

0.05 ml of standard calcium solution is pipetted into 0.5 ml of ammonia-buffer solution. 1 drop of indicator solution and 1 drop of magnesium complexonate solution are added and the titration carried out as under 1.

Constriction micropipettes of the Carlsberg pe have been used to measure off the umes of serum and calcium standard solun. All solutions should be contained in sels of resistant glass and should be conlled frequently for contamination by calm. Titration is to be carried out in good ht, preferably from a fluorescent lamp. The serum must not contain red blood ls, because of their high content of magium. For the same reason magnesium l be found erroneously high in hemolyl sera. The value of calcium determined er precipitation will not be influenced by nolysis.

CALCULATIONS

$$\text{Concentration of EDTA in mEq/L} = \frac{\text{Titration in ml}}{\text{Volume of Ca-standard (ml)}} \times 5$$

B. Sum of Ca and Mg in mEq/L (direct titration)

$$= \frac{\text{Titration in ml} \times A}{\text{Volume of sample (ml)}}$$

C. Calcium in mEq/L (titration after precipitation

$$= \frac{\text{Titration in ml} \times A}{\text{Volume precipitated (ml)}}$$

D. Magnesium in mEq/L = B — C.

Magnesium in serum averages 1.9 mEq/L (Stutzman and Amatuzio 1952). Subtraction of this value from B gives an approximate value for calcium which may be sufficiently accurate for most clinical purposes.

Calcium in mg per 100 ml = Calcium in mEq/L × 2.004.

Magnesium in mg per 100 ml = Magnesium in mEq/L × 1.216.

ACCURACY OF DETERMINATIONS

10 titrations were carried out on the standard calcium solution containing 5 mEq/L. Then solid $MgCl_2 \cdot 6H_2O$ was added to the standard, corresponding to 3.09 mEq/L. Calcium was precipitated from this solution and

Table I. *Titration of Ca-standard, Ca-standard+Mg and calcium precipitated from Ca-standard+Mg.*

	Mean of titrations in ml	Mean of deviations in ml	Deviations in mEq/L
Calcium standard (direct titration) ..	1.009	± 0.009	± 0.045
Ca-standard + Mg (direct titration) ..	1.632	± 0.008	± 0.040
Ca-standard + Mg (precipitated)	1.008	± 0.008	± 0.040

subsequently titrated. The results of direct titration and titration after precipitation were compared.

Similarly 10 titrations were carried out on a sample of serum directly, and on calcium from the same serum precipitated as oxalate. The results are tabulated.

Table II. *Direct titration of serum and titration of calcium precipitated from serum.*

	Mean of titrations in ml	Mean of deviations in ml	Deviations in mEq/L
Serum, direct titration	1.329	± 0.006	± 0.030
Calcium, precipitated from serum	0.983	± 0.013	± 0.065

As appears from these titrations, no loss of calcium occurs during precipation or subsequent manipulations. Mean of deviations for direct titration of serum and for calcium isolated as oxalate is approximally ± 0.05 mEq/L or ± 1 per cent of normal values for calcium in serum. Magnesium is determined by two titrations. Mean of deviations for determinations of magnesium therefore will be ± 0.1 mEq/L or approximately ± 5 per cent of normal values.

SUMMARY

A micromethod for the determination of calcium and magnesium in 0,05 ml of serum by titration with ethylene-diamine-tetracetate and the dye Eriochrome Black T as indicator is described. The sum of calcium and magnesium is found by direct titrat of serum. Calcium is determined after p cipitation as oxalate. Perchloric acid is u for decomposition of oxalate, whereby cium becomes directly titratable. Magnesi represents the difference between the titrations.

The method has shown sufficient ac racy, having a mean of deviations for cium of ± 1 per cent and for magnesium ± 5 per cent.

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PAPER CHROMATOGRAPHY OF PORPHYRIN PIGMENTS

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Nicholas & Rimington, in 1949, introdu- l a method for the separation of porphyrin gments by paper chromatography. The ethod is, however, relatively time-consu- ng as 12—14 hours are needed for an ana- is. The author was in need of a more pid analytical method, which was easy to ndle and could be used for preparative ork, and the procedure of Nicholas & mington was sought modified in different ys. It was found that the ascending met- d (Williams & Kirby 1948) was easier handle than the descending method of cholas & Rimington, and gave good sepa- ion of the diffent pigments in less than e hours. Saturation of the chromatogra- ic chamber with the eluent and ammonia fore the start of an analysis was found necessary, and the whole apparatus needed a complete set-up could be simplified. — the beginning the gas/liquid system used s the same as described by Nicholas & mington (1949). In a later paper Nicholas Rimington (1951) give further infor- tion on their method. A better separation porphyrins was obtained in a system ich did not contain ammonia. This, howe- , was not the case in the ascending met- d used by the author. The spots became ader and artefacts occurred. During the course of the investigation R. Kehl & W. Stich (1951) reported an ascending method using a system containing 2—6 lutidine/ water/ammonia. 2—6 lutidine is completely miscible with water up to 40° C, while 2—4/ 2—5 lutidine is completely miscible only at temperatures below 17° C. To work with completelly saturated 2—4/2—5 lutidine water mixtures at temperatures around 20° —25° C is inconvenient, as separation of the two liquid phases may occur and spoil the analysis. With 2—6 lutidine this problem does not arise, and this solvent has been preferred by the author.

METHOD

Uroporphyrin I methylester, coproporphyrin III methylester and mesoporphyrin ester[1] were hydrolyzed with 7 N HCl at room temperature for 36 hours. The hydrochloric acid was removed in a vacuum desiccator over sodium hydroxide pellets and the porphyrins dissolved in a small amount of 2 N NH_4OH and applied to the paper in this solution. The 3 and 5 carboxylated porphyrins used have been obtained from an investigation of experimental porphyria in rabbits, which will be dealt with in a separate publication. Watman filter paper No. 1 for chromatography was cut into sheets

[1] The test porphyrins have been obtained from Professor Rimington. The author is grateful for this courtesy.

20 × 25 cm and the porphyrins were put on the paper 2.5 cm from the shorter edge with a capillary blood pipette with a capillary of 10 mm.[3] The solution was allowed to creep out by the capillary activity of the paper. This application method is easy to handle, and the spots may be kept as small as desired. Usually the diameter of the spots was not allowed to become more than 0.5 cm. The distances between each spot were not less than 1 cm and no spot was applied closer to the longer edges of the paper than 4 cm. It was therefore possible to analyse at least 10 different porphyrin mixtures on one single sheet of paper. It is always necessary to apply markers on the paper as small variations in the Rf values may occur. The spots are dried with and air blast using air of room temperature, and the paper sheet is formed into a cylinder which is held together with capillary hooks or silk thread. The longer edges of the sheet must not touch each other as this may cause sideflow of the solvent mixture and spoil the chromatogram.

The cylinder is put into a Petri dish filled with a 2—6 lutidine/water mixture in the ratio 5/3 up to 0.5 cm from the bottom. In the center of the dish is placed a small beaker containing 25 per cent NH_3. The petri dish stands on a plane ground glass plate which is placed on a horizontal table. A glass cylinder ground at the open end is placed over the paper cylinder and the dish and the apparatus are left for 4—5 hours during which the solvent front will rise around 15 cm. The analysis should preferably be performed in the absence of light as the lower porphyrins are unstable to light in alkaline solution. Due to the relatively short time for a total analysis, however, this is not so critical in the procedure described as in the longer procedures of Nicholas & Rimington and Kehl & Stich. The paper cylinder is taken out and dried with the air blast and the chromatogram is developed under ultra-violet light. The author uses as light source a Philora lamp with a Woods filter.

RESULTS

It is found that the ascending chromatographic method is timesparing and very easy to handle. Nicholas & Rimington use either 2—4/2—5 lutidine saturated w water at the working temperature or t work at temperatures below 17° C. T author, however, has found in accorda with Kehl & Stich (1951) that 2—6 lutidi which is completely miscible with water to 40° C, is easier to handle and gives good separation as the 2—4/2—5 lutid system. In the ascending method it is fou that NH_3 vapor improves the results obt ned in contrast to the findings with the d cending method of Nicholas & Riming (1951). Kehl & Stich used ascending ch matography and a system containing 2- lutidine/water in a ratio 1/1 and NH_3 vap The paper used by these authors was "Fil paper No. 2043 b from Schleicher Schüll". They reported that the time nec sary for one analysis is 12—14 hours an slight streaking was observed. In the pro dure described "Wathman No. 1 filter pa for paper chromatography" was used, streaking was observed and the time nee for a complete analysis of five differ porphyrins was around 5 hours (Table With a ratio of the lutidine and water of

Table I. *Rf values of porphyrins obtai with 2—6 lutidine/water/NH_3 and 2—4/2- lutidine/water/NH_3 on Wathman filter pa No. 1 for chromatography.*

	2—6 L/H_2O 5/3	2—4/2— L/H_2O 7/5
Uroporphyrin	0.06(0.12)	0.02
5 carboxylporphyrin	0.42	0.30
Coproporphyrin ..	0.56	0.41
3 carboxylporphyrin	0.68	0.50
Mesoporphyrin	0.83	0.60

separation of the lower porphyrins appe-
d and uroporphyrin had a Rf-value of
. It was therefore necessary to decrease
amount of water and it was found that
atio of lutidine/water of 5/3 gave the best
aration. At lower ratios the Rf-values
reased for all porphyrins while at higher
ios the Rf-values decreased and streaking
urred. Uroporphyrin usually gave two
ts on the chromatogram. This has also
n reported by Nicholas & Rimington
951).

When working with 2—4/2—5 lutidine
author uses a ratio of lutidine/water of
at a temperature of $20 \pm 1°$ C. This
tem gives a satisfactory separation of the
phyrins. The Rf-values, however, are
er than in the 2—6 lutidine method des-
bed above (Table I), and separation of
liquid phases may occur and spoil the
omatogram. With smaller amounts of
ter the Rf-values decrease so much that
separation of the porphyrins is obscured.
nen working with 2—4/2—5 lutidine it is
ferable to work at temperatures below
° C.

SUMMARY

1. A simple and time-sparing procedure for the paper chromatographic separation of porphyrin pigments has been described.

2. 2—6 lutidine, which is completely miscible with water at temperatures up to 40° C, has been found to be easier to handle than the 2—4/2—5 lutidine used by Nicholas & Rimington (1949, 1951).

3. In contrast to the findings of Nicholas & Rimington (1951), a system containing NH_3 vapor is found to give better separation of the porphyrins.

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STUDIES ON EXPERIMENTAL PROTEINURIA I

ELECTROPHORETIC AND CHEMICAL INVESTIGATIONS OF THE AMOUNT AND COMPOSITION OF THE URINE PROTEINS IN CYTOTOXIC MASUGI NEPHRITIS IN THE RAT

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It seemed of interest, in the course of an investigation of Masugi nephritis in rats, to obtain accurate information on the amount and nature of the excreted urine proteins in various stages of the disease.

Since the fundamental works of Masugi (1933, 1934) a number of investigations have been published on this type of experimental nephritis. In few of them has the rat been used as a test animal (Smadel, *et al.* 1936). Two groups of workers only made use of quantitative urine protein estimations (Smadel *et al.* 1936—1939, Knowlton *et al.*, 1950). No fractionation studies have been made. The results indicate that the proteinuria generally reaches its maximum rapidly at the beginning of the experiment and then slowly decreases. The rapidity of the decrease depends on certain factors, e. g. the strength and dosage of the renal antiserum and the protein content of the diet.

A great many investigations have been published on this problem in clinical proteinuria. Among them the studies concerning the electrophoretic method are of special interest (Hesselvik, 1939, Luetscher, 1940—1947). From these we learn that all the serum fractions are found in the urine, but no serial investigations of patients seem to have been made. Salting out studies dur the course of proteinuria have been perf med (Bing, 1936, Berglund, 1936), but in case from the very beginning of the r disease. On this point there is obvious lack of information.

Observations on clinical proteinuria f this laboratory (Laurent, 1951) show f tuations in the hexosamine and hexose tent of the albumins and globulins in serum and urine. Fairly great variation the levels of protein-bound carbohydrat the urine have been found in different k of proteinuria. The results also indic that variations exist in the urine pro sugar values in different stages of the r disease.

Fahr (1936) among others, claims tha clinical and histological picture of Ma nephritis has many features resemb some human types of renal disease, b must be said that they do not quite cover specific, well-defined human renal diseas is generally accepted, however, that the of a renal antiserum is to be preferre most of the other nephrotoxic agents cause of its relative specificity and range of dosage.

The albino rat seems to be well suited for kind of experimental work, where repro-ibility and standardization of the experi-nt is of decisive importance.

By comparing the results from repeated ntitative urine protein determinations, electrophoretic distribution of protein ctions in the urine, and the urine protein osamine values during experimental renal ease in rats, we hoped to obtain some neral information regarding the develop-nt and course of proteinuria.

EXPERIMENTAL

Ten male albino Wistar rats were used in the sent series. They had an average body weight 264 g and an average protein excretion of 0.2 /hour at the beginning of the experiment.

The antiserum was prepared according to Smadel Farr (1937) and has been used before in this oratory (Laurent, unpublished). The rabbit o. 5) was injected intraperitoneally 32 times h ten ml of a 20 per cent w/v sterile (bacterio-cally tested through cultivation) suspension of od-free rat renal tissue in physiological saline in course of 86 days. The rat kidneys were minced Latapie tissue mill. The rabbit was bled under t ether anaesthesia from the carotic artery 34 s after the last injection.

The renal antiserum thus obtained was injected avenously into the tail vein of the rat using l of the serum diluted 1 : 5 with physiological ne. All rats received same amount of serum. anaesthesia was used.

The rats were kept in separate wire cages over unnel for 20 to 24 hours for urine collection. ring this time they drank a liquid (modified ording to Addis *et al.*, 1949) consisting of 7 g IDAMIN,[1] 150 g sucrose, 4 g NaCl and 2 ml amin-B-comp. (Medica) per liter. 30 ml of this id was measured in a drinking bottle fixed over nall vessel in the cage to prevent dripping into the urine receptacle. Except on two occasions all the liquid offered was drunk up by the animals. This diet did not influence the protein excretion in control animals.

The rats were divided into 2 groups of 5 animals, placed in the collecting cages on alternate days. This was done because we had found that healthy rats suffered from profuse, sometimes hemorrhagic diarrhea soon after 1 week on liquid food. Hence the test animals were placed on their ordinary flour-casein basal diet[2] every other day during which time no urine was collected.

The animals were in good condition throughout the experiment and showed no visible signs of infection. The average body weight rose during the experiment from 264 to 271 g. The room temperature varied considerably during the experiment (from + 13° C to 20° C) but the rats were kept more than 1 month under the same conditions before being injected.

The urine volume was measured to an accuracy of ± 0.5 ml. The diuresis was expressed as ml per hour. Toluene was used as a preservative during the collection.

Total proteins in urine were measured by means of the biuret reaction using the modification of Laurent and Lamberg (1947) adopted for the Unicam spectrophotometer (wave length 530 mμ). The protein was precipitated at 7 per cent w/v concentration of trichloracetic acid, centrifuged, dissolved in NaOH and treated with the copper sulphate-sodium citrate reagent. The centrifuge tube was graduated at the 10 ml level so that the whole procedure could easily be carried out in the same tube. A standard curve was prepared by using dilutions of serum with a known protein concentration and it was found to be straight for protein values ranging between 0 and 18 mg. No difference was found between protein values obtained through direct estimations or trichloracetic acid precipitations of the protein from the standard solution. The proteinuria was expressed in mg per hour.

The serum proteins were determined by the same method directly from 0.1 ml of serum.

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[1] A casein hydrolysate kindly supplied by Orion, Helsinki.

[2] Rye flour 7800 g, wheat hull 2400 g, casein 1080 g, NaCl 60 g, $CaCO_3$ 60 g; carrots and Swedish turnip ad libitum.

Electrophoresis. The collected urines were kept refrigerated at —4° C until the investigation was made (1 to 12 weeks later). Before this, each sample containing less than 1 per cent protein was concentrated to a protein concentration of 1 per cent by dialysis against dextrane solution. This was followed by dialysis against a phosphate buffer pH 7.8, ionic strength 0.15, for 3 days. The electrophoresis was made using a Tiselius apparatus with two centre cells and the optical arrangement of Svensson. The buffer used was that mentioned above, temperature 0.7° C, migration time 135 minutes. The diagrams of the descending side were magnified fourfold and the areas under each peak measured with a planimeter. From these the relative concentrations were calculated. The serum samples were dialysed against the same phosphate buffer for 2 days, diluted to a protein concentration of 1.5 per cent and analyzed using the same technique.

The hexosamine estimations were performed from dried urine protein preparations. The protein was coagulated by boiling with an acetate buffer at pH 4.2 and centrifuged down. The precipitate was then suspended in 70 per cent v/v ethanol, kept at 75° C for 15 minutes and centrifuged. This procedure was repeated twice after which the precipitate in the centrifuge tube was left to dry at 50° C overnight, pulverised, and placed in a vacuum desiccator for storing. Before weighing the powder was kept 3 hours at 95° C to achieve constant weight, and returned to the desiccator, care being taken to avoid contact with the air before weighing. 25 mg of the powder was then hydrolysed in a test tube, closed by melting, in 2 N HCl at 100° C for 20 hours. The colorimetric hexosamine estimation was then made from the neutralized hydrolysate according to the Elson-Morgan method as modified by Blix (1948). The values were expressed as gram hexosamine per 100 g protein and not corrected for ash estimations.

The ash estimation gave 0.64 per cent or 6.4 mg of ash per gram of a pooled lot of rat urine proteins.

The benzidine test was performed only when the urine seemed dark.

For the histopathological investigation the kidneys, liver, spleen, adrenals and the left ventricle of the heart were fixed in neutralized 10 per v/v formaline for 24 hours. Hematoxylin-Gieson staining was used throughout. The exam tion was performed at the end of the experi when all animals were killed.

RESULTS

The follow-up examinations were m over a period of 43 days from the inject the animals being followed more closely the first 3 weeks.

Urine investigations.

A. Proteinuria and diuresis. (Figs. 1 Table I). — A somewhat surprising fact that 2 of the animals (Nos. 1 and 10) did develop proteinuria after being injec Animal No. 4 reached the pathological l in the first few days, but the urine pro level returned to normal again in a w One of the animals (No. 5) did not bec proteinuric until the 5th day after inject All the others revealed intense protein on the first two days. The maximum pro excretion was noted during the first second days. The first maxima were usu followed by one to four smaller peak intervals of 4—6 days. The protein never returned to normal values in the days of the experiment, but it decre slowly. Observations before the injectio the antiserum show individual and diffe levels of protein excretion varying betw 0.07 and 0.77 mg per hour. Changing f the usual (0.16—0.56 ml per hour) to a her (0.85—1.25 ml per hour) diuresis did not affect the normal proteinuria to great extent.

The diuresis remained fairly constant can be seen from Table I, except for

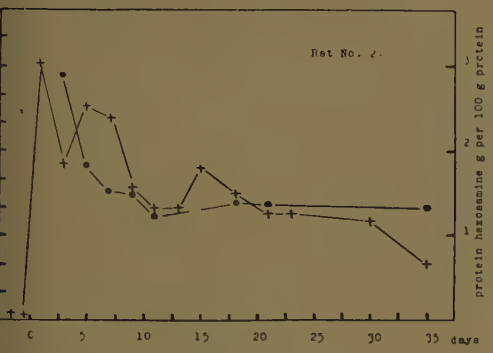

Fig. 1.

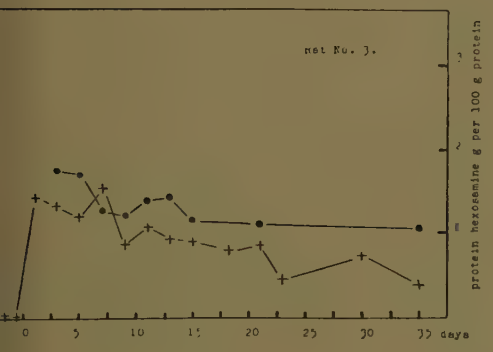

Fig. 2.

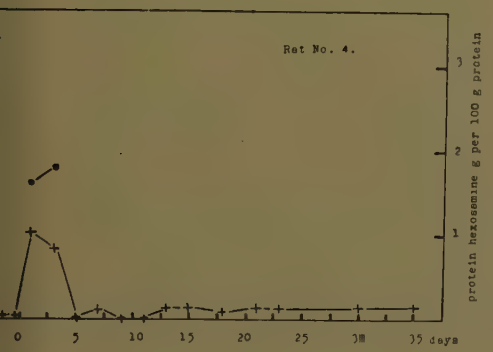

Fig. 3.

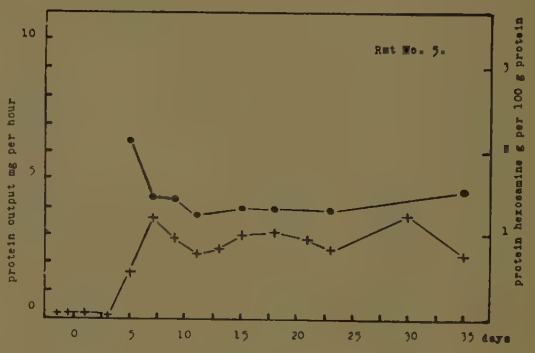

Fig. 4.

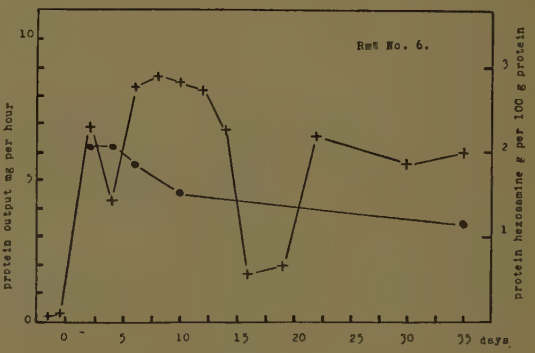

Fig. 5.

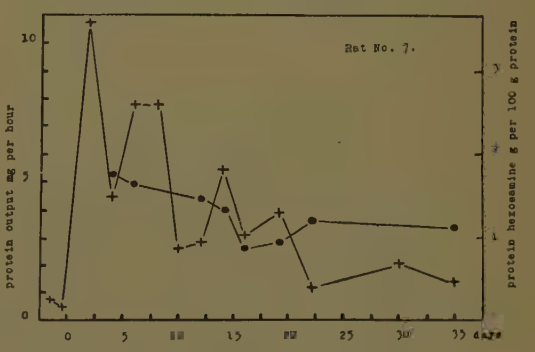

Fig. 6.

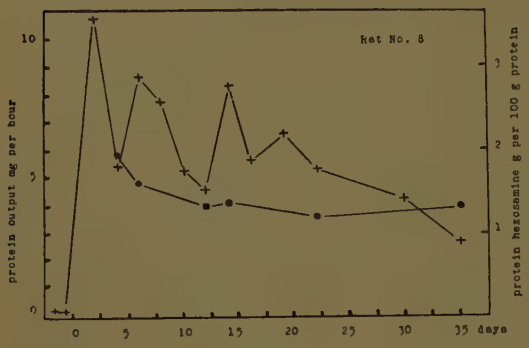

Fig. 7.

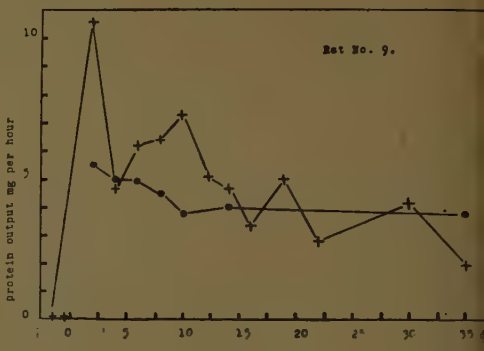

Fig. 8.

Fig. 1—8. Protein output in urine and hexosamine content of excreted protein by 8 rats two day before and 35 days after an injection of renal antiserum prepared according to Smadel and Far

+——+ protein output ●——● hexosamine

animal (No. 6). It revealed an oliguric phase between the 16th and 19th days. The influence of the diuresis on the proteinuria can be seen in the two experiments carried out respectively on the 35—36th and 41—43rd days. (Fig. 9). During the first experiment the animals received 10 ml of the fluid drink, they then stayed overnight in the c lecting cages, during which time they w given 100 ml of fluid. In the second expe

Table I. *Diuresis expressed as ml/hour of 10 Wistar rats 2 days before and 30 days after an injection of renal antiserum.*

Day	Rat No.									
	1	2	3	4	5	6	7	8	9	10
−2	.33	.31	.25	.36	.22	.17	.56	.42	.31	.22
−1	1.13	1.17	1.02	.96	1.13	.88	1.17	1.25	1.13	.85
+1	1.08	.95	.55	1.18	.92	1.58	.61	1.37	1.45	.79
3	1.27	1.21	1.17	1.15	.96	1.58	1.27	1.32	1.41	1.50
5	1.37	1.42	1.21	1.29	1.32	1.10	1.30	1.23	1.33	1.43
7	1.26	1.13	1.20	1.20	1.29	1.31	1.39	1.00	1.24	1.42
9	1.42	1.37	1.18	1.21	1.60	1.10	.65	1.30	1.25	1.03
11	1.35	.95	1.16	1.30	1.28	1.21	1.16	1.34	1.40	1.37
13	1.44	1.50	1.05	1.22	1.39	1.29	1.61	1.71	1.24	1.27
15	1.11	1.14	.96	1.22	1.26	.30	1.20	1.55	.80	1.40
18	1.15	1.37	1.07	1.17	1.39	.18	1.13	1.43	1.36	1.10
20	.95	1.18	1.00	1.07	1.10	1.23	.71	1.40	.77	1.10
30	1.23	1.06	1.22	1.38	1.33	1.01	1.08	1.53	1.28	1.21

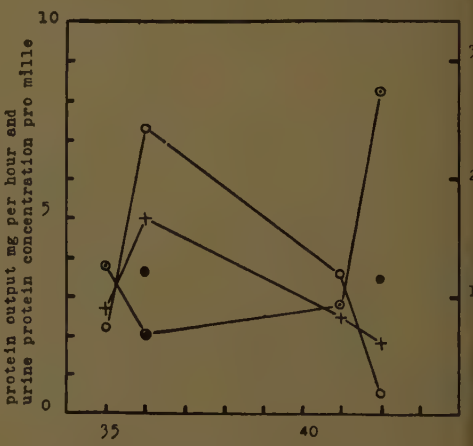

Fig. 9. The effect of various diuresis levels on proteinuria (rat No. 8). For details see in t

○——○ diuresis ml/hour
+——+ protein output mg/hour.
⊙——⊙ urine protein conc. promille
● ● protein hexosamine g/100 g dry protei

ent the animals stayed 3 days in the collecng cages and were given 30, 10, and 100 l of the liquid, respectively. In both exeriments a close parallelism can be observed etween the diuresis and the amount of exeted protein. When the diuresis is constant, it is almost throughout the experiment, relationship is discernible between protein utput and water excretion.

B. Electrophoresis. A total of 20 samples f urine were examined electrophoretically. he results of these analyses are presented Figs. 10—13, and Table II. In general, e separation of proteins to well-defined eaks is much better in rat urine than in rum. Of the protein excreted in urine uring the early phase of the disease 70 to per cent is albumin, the rest being divided out equally among the three globulins. As general feature the relative albumin values ve a tendency to diminish when the protein xcretion decreases, whereas the relative nount of γ-globulin increases. To illustrate is observation more clearly we have plotted e relative concentrations of both albumin d γ-globulin against the urinary total proin values for the whole material examined Figs. 14 and 15).

The α- and β-globulins fluctuate between ther small limits, showing no definite riations. A probable increase in α-globulin d a decrease in β-globulin during the dise e is observable, but this is not convincing. addition, in 3 cases there was a small but stinct component moving faster than albuin, with a relative concentration of about per cent. During the later course of the sease this component diseappeared. This action is in all probability identical with the mponent *f* observed in rat serum.

C. *Protein hexosamine.* The amount of hexosamine in total *serum* protein in a pooled preparation of serum from 10 normal Wistar rats of the same age and sex as the test animals was found to be 2.69 g per sent. The serum proteins were prepared in the same way as the urine proteins, except that 3 additional treatments with ether-ethanol $\overline{aa}$ were employed. This value is in general higher than the hexosamine values found in the urine preparations. These are presented in Figs. 1—8 together with the protein excretion levels. 8 of the 10 animals were examined, because the other 2 had too small amounts of protein for an exact determination. In all animals there occurs a decrease of the hexosamine content of urine proteins during the first two weeks after injection. After this hexosamine values remain at a rather constant level. The values seem to follow the decreasing proteinuria to a certain extent. The variations in protein excretion induced by various diuresis levels do not, however, influence the hexosamine values (Fig. 9).

D. *The benzidine test.* The benzidine test for detection of blood in urine was always negative. The urine sediment was not examined.

Serum investigations.

The serum of all 10 animals used in this investigation was analyzed electrophoretically on the 44th day after the injection of renal antiserum (at the end of the experiment). The total serum protein values and percentile distribution of various components are shown in Table III. The electrophoretic data from 6 normal rats of the same strain are shown in Table IV. In general the sepa-

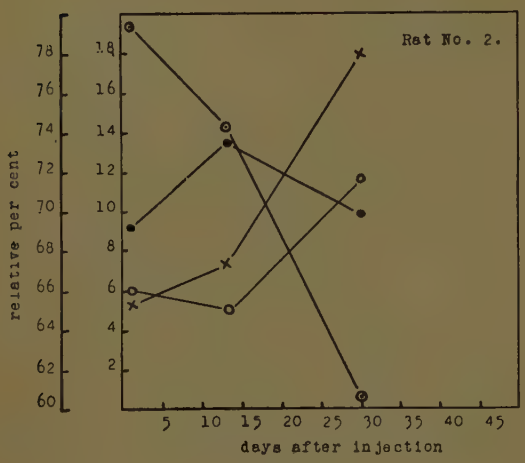

Fig. 10.

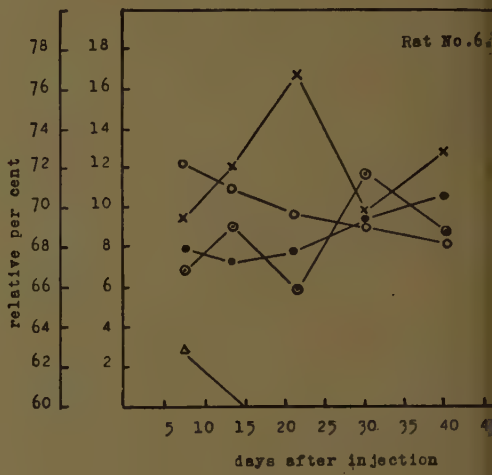

Fig. 12.

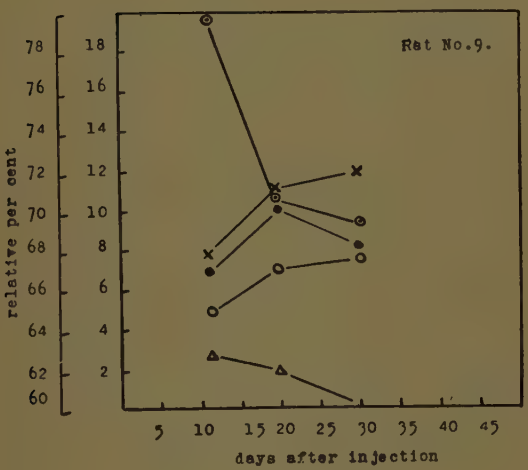

Fig. 11.

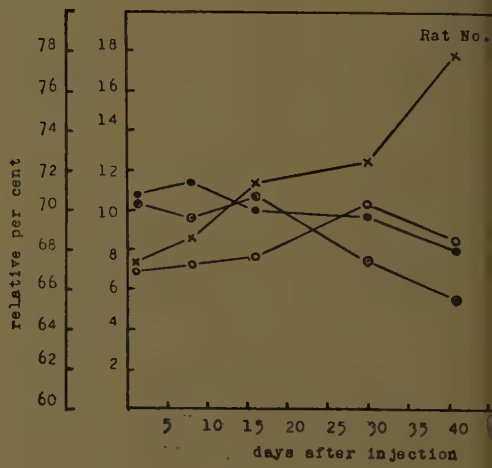

Fig. 13.

Fig. 10—13. Urinary excretion of various protein fractions by rat during the course of proteinuri induced by one injection of renal antiserum.

⊙——⊙ albumin
○——○ α-globulin
●——● β-globulin
×——× γ-globulin
△——△ "fraction f"

able II. *Electrophoretic Analysis of ine Proteins of 3 Wistar Rats after Injections of Renal Antiserum.*

Rat. No.	Days after injection	Percentile composition			
		Albumin	Globulin		
			α	β	γ
3	1	76.4	6.7	12.4	4.5
5	30	71.5	11.1	7.6	9.8
7	2	69.7	9'5	12.5	8.3
7	8	74.8	10.3	6.1	8.8

able III. *Electrophoretic Analysis of rum Proteins of 10 Wistar Rats 44 Days er the Injection of Renal Antiserum prepared according to Smadel and Farr.*

at o.	Total protein g per 100 ml	Percentile composition					
		f	Albumin	Globulin			
				α_1	α_2	β	γ
1	6.85	—	50.3	3.3	10.8	17.3	18.3
2	6.00	—	41.5	27.8		15.1	15.6
3	6.75	3.1	51.5	13.0		15.7	16.7
4	6.60	—	46.5	8.0	8.2	21.1	16.2
5	6.25	—	45.6	21.3	6.6	12.7	13.8
6	4.95	6.3	49.7	22.4	6.4	9.4	5.8
7	6.35	2.0	43.4	11.0		36.7	6.9
8	5.00	-	51.0	16.4		17.1	15.4
9	6.20	6.3	49.7	14.5		16.6	12.9
0	5.80	2.9	54.8	16.0		15.4	10.9
an	6.10		48.4	18.6		17.7	13.3

ion of the individual protein components rat serum is rather poor and highly variafrom one animal to another. Two disct globulin fractions were observed in all a examined, representing the β- and γbulins of human serum (as far as mobiis concerned). In addition a third globufraction was always seen between albu-

Table IV. *Electrophoretic Analysis of Serum Proteins of 6 Normal Wistar Rats.*

Total protein g per 100 ml	Percentile composition					
	f	Albumin	Globulin			
			α_1	α_2	β	γ
6.07	8.1	54.6	6.8	5.0	11.5	14.0
6.17	—	61.3	10.3	2.7	11.8	13.9
5.77	—	54.5	6.9	4.9	15.8	17.9
6.14	+	55.7	9.3	5.5	14.5	15.0
7.25	1.5	53.4	12.5		19 5	13.1
7.00	—	54.2	8.1	6.0	16.8	14.9
Mean 6.40		55.6	13.0		15.0	14.8

min and β-globulin, but its separation from albumin was highly variable. In many cases two more or less distinct peaks, designated α-1 and α-2, existed between albumin and β-globulin. A small component moving faster than albumin, was first described by Deutsch and Goodloe (1945) in rat serum (designated by them as "component f"). In the present series this peak was discovered in 3 normal and 5 nephritic sera. In all the animals whose urine contained a fast-moving peak during the early phase of the disease, component f was present in serum (Nos. 6, 7, and 9). The differences in the serum protein pattern between the nephritic and the control group are relatively small. No significant difference exists in the total protein, β-globulin and γ-globulin levels. Animal No. 7 has a surprisingly high β-globulin, the cause of which remains obscure. The albumin values in general show a small decrease from the normal level, whereas α-globulins are distinctly elevated in 3 cases (Nos. 2, 5, and 6).

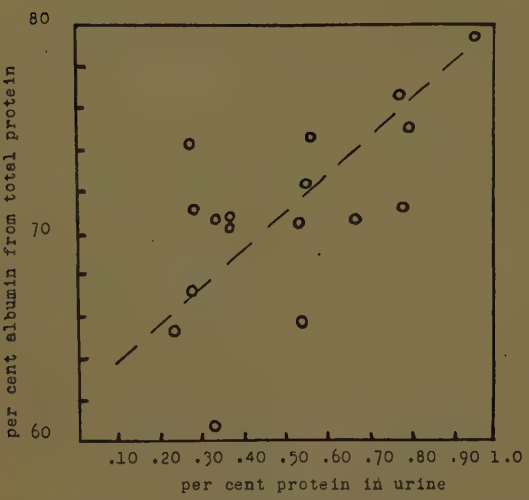

Fig. 14. Correlation between the urinary excretion of albumin and total protein in renal antiserum-induced proteinuria.

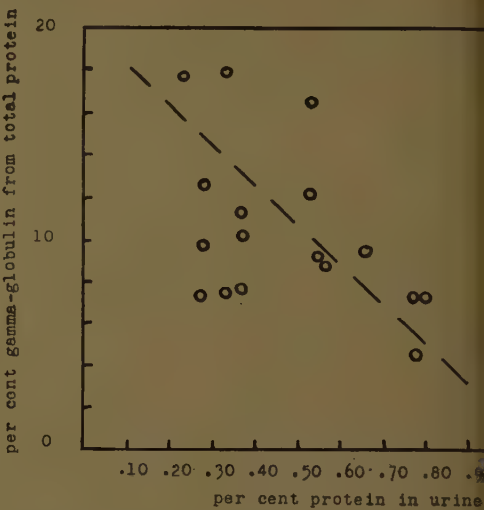

Fig. 15. Correlation between the urinary excre of gamma-globulin and total protein in renal a serum-induced proteinuria.

Gross anatomy and histopathological investigations.

Macroscopically there was nothing pathological to be noted. No signs of ascites. The renal capsula was removable without difficulty and the surface of the kidneys was quite smooth.

The liver, spleen, adrenals and the left ventricle of the heart showed nothing histologically abnormal in any of the 10 animals.

The microscopical preparations were kindly examined by Professor Ivar Wallgren, M. D. The spleen, adrenals and the left ventricle of the heart showed nothing histologically abnormal in any of the 10 animals. No. 7 had small, diffusely demarcated necrotic areas in the liver, also No. 9, but to a lesser extent. The other animals showed intact liver tissue. In the kidney sections some of the glomerules showed anemic capillaries. The endothelial cells in the capill wall in some cases stood closer together t normally. Some of the glomerules seeme be intact. The renal changes varied betw rather narrow limits in all of the 10 anim but all kidney sections showed a patholog picture. No tubular changes or cast for tion in the lumina could be observed w certainty.

DISCUSSION

The purpose of the present investigat has been to shed light on the amount composition of urine proteins excre during Masugi nephritis in rats, and, if sible to obtain information on the mechan of proteinuria.

It should be noted that no renal clear investigations have been made. The diur experiments and the general course of

periment give us, however, some informa-
on on renal function.

Oliguria (i. e. excretion of less than one
lf of the amount of fluid consumed)
curred only 6 times (animal No. 3 on the
t day, No. 6 on the 19th day and No. 7 on
e 2nd, 10th and 36th days). Twice the
imals refused to drink the whole amount
fluid offered. It can be stated that the
dneys were able to adapt themselves to the
nount of liquid consumed by the rats, bet-
een 10 and 100 ml during 20—24 hours.
o density measurements of the urine have
en made. This means we cannot judge
jectively whether the urine was isostenuric
not. At a low diuresis level the urine was
rk, at higher levels light colored.

This would suggest that the function of
e renal tubules at the end of the experi-
ent was possibly not very much decreased.
he oliguric attacks mentioned show that
ere really has been a transitorily decreased
nal function. According to Bing (1936)
is is a sign of tubular restriction of the
uresis, because of the inverse ratio of diu-
sis to the concentration of proteins in
ine.

The tubules were apparently able to
ncentrate and dilute the urine throughout
e experiment. But it must be added that in
ostenuria the urine volume would have re-
ected the amount of fluid consumed, the
dney serving merely as a filter: How-
er, Bing's statement is of great value
d encourages us in our theory that the
bular function was intact or nearly intact
cause, had the diuresis depended on glome-
lar regulation only, it would also have run
rallel to the protein values.

In the above-mentioned forced diuresis
periment we note that without any excep-
tion both the proteinuric and the non-pro-
tein-uric animals show a clear parallelism
between water output and the mg/hour pro-
tein values. This means that *more protein is eliminated when the glomerular filtration increases.*

We have not found this phenomenon de-
scribed in the literature. Bing (1936) states
that the calculated average protein content in
the glomerules is unaffected by variations in
"filtration" and diuresis. (We have not, as
Bing did, carried out creatinine-clearances
in the present series, but have planned them
for future work). It seems to us that the
proteinuria decreases and increases parallel
to water output, instead of remaining at a
constant level.

The fact that greater variations in diuresis
occurred in only a few cases (animal No. 6)
indicates that there cannot have been any
significant blocking of the glomerules or
any greater tubular damage during the dise-
ase. The proteinuria can be explained as ex-
plained as resulting either from an increased
glomerular permeability, a blocked tubular
reabsorbtion of proteins or both (Smith
1951).

The histological picture at the end of the
experiment shows intact tubules in all ani-
mals but marked, though slight, pathological
changes in the glomerular structure. This
would at least indicate that there may
have been increased glomerular protein per-
meability, possibly during the whole experi-
ment. Nothing can be said about the rôle of
the tubules.

The electrophoretic analyses and hexo-
samine estimations show that the composi-
tion of the urinary proteins undergoes cer-

tain characteristic changes during the course of the experimental renal disease. These include a decrease in the relative albumin values and in the hexosamine content of urine proteins, and an increase in the relative γ-globulin values.

If we regard the amount of the proteinuria at a constant diuresis level as a measure of the existing renal lesion, we arrive at the conclusion that the greater the damage the greater the albumin excretion and the smaller the γ-globulin excretion (Figs. 14 and 15). This is rather surprising and difficult to explain merely on the basis of the theory of the glomerular permeability of protein. Indeed, if protein reabsorbtion does occur in the tubules, it can be imagined that the albumin, having a smaller molecular size, is more readily reabsorbed than the γ-globulin during recovery from the disease. This hypothesis is further supported by the observation of Bing and Harboe (1951) that fetuin, a protein with a smaller molecular weight (51000) than albumin, is retained in the blood. Another view is that the glomerular permeability of a given protein is also determined by the shape and charge of the protein molecule (Marshall & Deutsch, 1950, Bing & Harboe, 1952). Especially the assumption of the effect of the charge might explain the excretion of γ-globulin.

The significance of the lowering of the hexosamine content of urinary proteins after the acute phase is difficult to interpret. There is no correlation between the hexosamine values and the excretion of α- and β-globulins, which are known to have the highest carbohydrate content (Blix, Tiselius & Svenssson, 1941). As an explanation it could be suggested that some fraction contains a protein rich in carbohydrate, amount of which then decreases.

The fraction f, which has a greater m bility than the albumin at pH 7.8, can p bably be a carbohydrate-rich mucoprote

As far as we know, no systematic studi of this kind have been made on the compo tion of the urinary proteins in clinical re diseases. Moreover, for practical reasons, is most often impossible to following a cli cal case from the very beginning of disease. Investigations of the electrophore distribution of urine proteins reported in literature comprise a few cases only.

Longsworth & MacInnes (1940) studi 2 nephrotic urines electrophoretically a found them to resemble the normal ser protein pattern. The mobility of urine alb min was smaller than that of serum albumi In the 7 cases presented by Luetsch (1940), albumin accounted for about 90 p cent of the urine proteins in nephrosis a 70 to 80 per cent in the terminal phase glomerulonephritis. These results were co firmed by Malmros & Blix (1946). Acco ding to Luetscher (1947), the relative p portions of various protein fractions in ur are determined by the composition of plasma and the permeability of the injur nephrons; thus in the presence of a const renal lesion it is possible to change the cretion of a fraction by changing its co centration in the plasma. In general, albumin is most readily excreted during nephrotic phase of clinical glomeruloneph tis (when the protein excretion also is kno to be greatest), while toward the termi stage the relative amount of γ-globulin creases (Blackman & Davis, 1943). T

re is a rough parallelism between total tein and albumin output, but it must be ed that these clinical cases were rapidly gressing ones whereas our experimental al lesions were regenerating (diuresis, teinuria, histological picture of the kid- s at the end of the experiment).

nteresting electrophoretic studies on clini- proteinuria have recently been reported Parviainen et al. (1951), who investi- ed urinary protein fractions in the toxe- s of pregnancy. They found the relative centage of albumin increased with the ical severity of the disease. In very mild es only about 30 per cent of total pro- ns was albumin, while the concentration α-globulins was extraordinarily high. The hors do not endeavour to interprent their lings. There is a disproportion between presumable mean molecular weight of nary protein and the severity of renal on.

There are indeed still other factors that st be taken into account. Later investi- ions into Masugi nephritis (Swift & adel, 1937, Pressman & Keighley, 1948, oman et al., 1949) show that the glome- es act as antigens during the immuniza- n of the rabbit and that the antibodies in renal antiserum are fixed to the glome- es when injected into rats. Kashibawara 39) claimed that immunization with iso- d tubules only gives rise to antibodies ich act on the tubules. According to ihler et al. (1951) the glomerules dama- by anti-renal antibodies cause secondary igen-antibodyreactions between the glo- rules and auto-antibodies. From clinical estigations (Schwentker & Comploier, 9) we know that in human scarlet fever, antikidney antibodies are found in the blood of nearly all of the patients.

The periodically occurring maxima of proteinuria found in this experiment could theoretically reflect some antigen-antibody reaction at the glomerular membrane (or in the tubule cells) of the rat, giving rise to an increased glomerular permeability (or a blocking of the absorption of proteins in the tubule cells).

The amount of antiserum (0.2 ml) used by the authors is rather small compared with the amounts mentioned in the literature. Its activity, however, seems to be relatively great: a dose of 0.3 ml given intravenously to a rat weighing about 250 g, killed it in 4.5 minutes. The whole does was always given in a single injection to avoid formation of anti-antibodies resulting from repeated injections.

On the whole the experimental renal disease reported in this paper has been of a rather benign nature. There was no hematuria, no edema, the rats were in good condition, only small changes occurred in serum proteins and the histological findings in the kidneys at the end of the experiment were slight. These observations show a clear parallelism with the result obtained by Smadel (1936) and Smadel & Farr (1937).

We have been able to collect some data on the urine proteins, but it has not been possigle to shed light on the mechanism of proteinuria. We do think, however, that the Masugi type nephritis might serve as a useful tool for investigations on this problem. For this purpose, renal clearance tests and more subtle histopathological examinations examinatins ought to be added.

SUMMARY

Experimental proteinuria was induced in 10 rats receiving equal amounts of a renal antiserum prepared according to Smadel and Farr. The course of the disease was followed for 43 days, after which time the animals were killed. The following results were obtained:

1. Seven of the animals developed a severe proteinuria which reached its maximum during the first 3 days after the injection and then slowly decreased but did not reach the normal level by the end of the experiment. One animal reached the normal level of proteinuria in a week, the other two animals did not develop any marked proteinuria at all. The proteinuria showed parallelism to water output in forced oliguria and polyuria. Oliguria occurred 6 times in all in three of the animals. At the end of the experiment all of the animals showed an intact diuresis regulating power.

2. The electrophoresis at the end of the observation time indicated rather small changes in serum proteins: a small decrease of albumin in most cases and a distinct increase of α-globulin in three cases. The electrophoretic examination of urine showed that during the early phase of the disease 70 to 80 per cent of excreated protein is albumin, the rest being divided equally among the three globulins. As a general feature the relative albumin values have a tendency to diminish with the protein excretion, whereas the relative amount of γ-globulin increases. In three cases a small but distinct component was seen, which moved faster than the albumin. During the later course of the disease this component disappeared.

3. The total urine protein hexosamine values showed a decrease in all cases during the first two weeks, after which they rem ned at a nearly constant level (1.1 to 1 per 100 g of protein). There seemed to no correlation between hexosamine le and the α- and β-globulins in the urine.

4. The histopathological investigation the end of the experiment showed a slig changed glomerular structure in all of animals. No tubular alterations were s

Problems connected with the mechan of proteinuria in the Masugi type of exp mental renal disease are discussed.

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SENSITIZED CLOTTING TIME AS A CLINICAL METHOD

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In recent years the use of substances which reduce the capacity of the blood to coagulate has gained increased popularity in treatment of thrombo-embolic diseases. However, treatment with anticoagulants, such as dicumarin, for instance, which prevents prothrombin synthesis in the liver, requires repeated checking of the prothrombin content of the blood. Quick's test has to date been the commonest method for determining the prothrombin content of the blood. It is not always reliable, however, and a well equipped laboratory is required for the determinations. Rosenthal (1949) describes an *in vitro* method with blood sensitized with heparin for determining the clotting capacity of the blood. With this method de Takats (1951) determined the coagulation rate in various conditions and during treatment with anticoagulants and compared the results obtained with Rosenthal's method with the prothrombin content of the blood. He found congruity good between the two methods; the sensitized clotting time was an even more sensitive test, indicating threatening hemorrhage with greater clarity. The knowledge of the clotting mechanism has now been greatly extended and new methods presented for routine determination of the prothrom content of the blood. Owren's studies sh that dicumarin reduces not only the p thrombin content of the blood, but also proconvertin content. (Owren & Aas, 195 On the basis of these results he worked o method for determining the prothrom percentage of the blood. The sensitized c ting time and the prothrombin percenta as determined by Owren's method, w compared in order to establish the value the former as a bedside method.

METHOD

Five micrograms of heparin in 0.1 cc phy logical sodium chloride is placed in a 13 × 100 test tube. One cc of blood is added. The tub corked and, after gentle inversion twice, is allo to stand for 12 min., after which it is inve every half-minute. The coagulation rate is reco from the moment the network of fibrin bec visible on the walls of the test tube. The proth bin percentages was determined at the hos laboratory by Owren's method. Blood sample determination of prothrombin content and clo time were taken at the same time and with the needle.

The series comprises cases treated phenylindandion and controls which had been given anticoagulants.

RESULTS

The clotting time in the controls was 12 to minutes (Table I).

Table I.

Diagnosis	Clotting time	Pro-thrombin
1. Ulcus ventriculi ..	13 min.	86 %
2. Hepatitis subchron.	15 »	63 »
3. Anaemia normochrom	14 »	78 »
4. Infarctus pulm.	12 »	78 »
5. Infarctus cordis	12 »	130 »

In Case 2, diagnosis: hepatitis, the clotting ne was the longest and the prothrombin ntent reduced. In the other cases the pro-rombin content was within the normal nits. The normal coagulation time deter-ned by this method thus seems to be 12 to

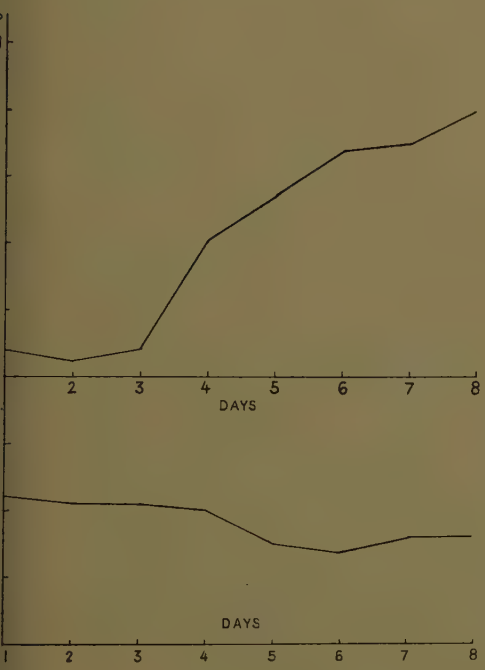

Fig. 1.

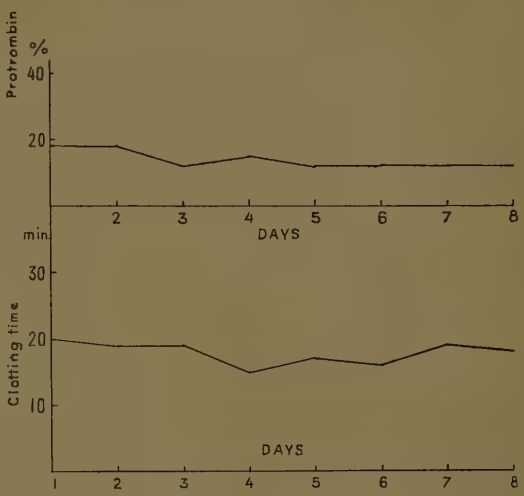

Fig. 2.

14 minutes. The following curves were obtained for 3 patients with infarctus cordis, subjected to phenylindandion therapy.

Fig. 1 shows a case in which the clotting time was determined towards the end of treatment. The prothrombin percentage rose after termination of treatment on the third day. The curves for clotting time and prothrombin content agree fairly well. In the first three days the prothrombin percentage was < 10 (a level indicating the risk of hemorrhage). The lotting time was 21 to 22 minutes during this period.

Fig. 2 shows a case with the prothrombin percentage constantly at the level desired in treatment of thrombo-embolic diseases. The clotting time varied from 15 to 20 minutes.

Fig. 3 shows a case in which treatment did not result in the desired prothrombin level. Yet the clotting time varied as in the former case.

There was some congruity between the curves for sensitized clotting time and pro-

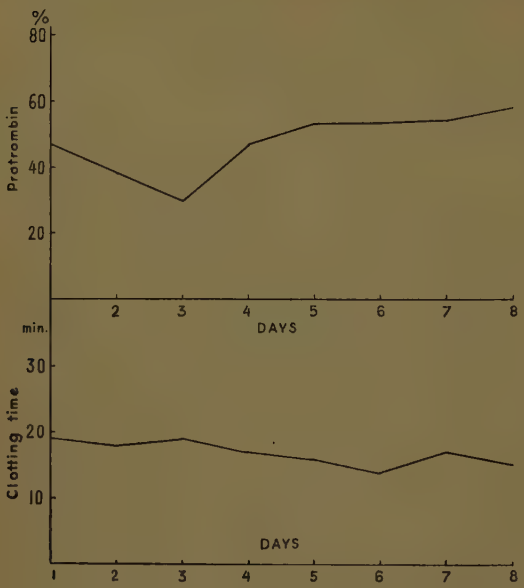

Fig. 3.

thrombin content, yet the difference between the values obtained, when the risk of hemorrhage was present and the normal values is so small that the use of sensitized clotting time as an indicator in treatment with anticoagulants must be considered of no av (Cf. Fig. 2 and 3). The advantage of a qu bedside method for determination of the c gulation power of the blood in treatm with anticoagulants is evident, but the ab method does not meet modern requireme

SUMMARY

The so-called sensitized clotting time compared with the prothrombin content the blood as determined by Owren's meth The test was not reliable enough for cont in anticoagulant therapy.

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NVESTIGATION OF VISCOSITY AND HYALURONIDASE HIBITORS IN JOINT EXUDATE AFTER INTRA-ARTICULAR CORTISONE INJECTIONS

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ıe permeability of synovial membrane e talocrural joints of rabbits for phenol- ıonphtalein is increased by intraarticular tion of hyaluronidase, but reduced by utaneous cortisone injection (Seifter, ler & Begany 1949).

rheumatoid arthritis the synovial mem- e has an increased permeability for ıal and acquired antibodies in the blood unsell, Wrigley, Highton & Holt 1950). viscosity of the joint exsudate in rheu- id arthritis is lower than that of synovia ormals (Robertson, Ropes & Bauer), and the degree of polymerisation of yaluronic acid in such exudate is like- lowered (Ropes, Robertson, Rossmeisl, ody & Bauer 1947).

cording to Seifter et al., the permeability e synovial membrane might to a large ee be regulated by the hyaluronidase- ıronic acid system. An imbalance in this m seems plausible when the permeability creased, as in joints with exudate in natoid arthritis.

e lowered viscosity of joint exudate can creased by intra-articular injection of cortisone. *In vitro* no effect of cortisone on the hyaluronidase-hyaluronic acid system has been demonstrated (Faber & Schmith 1950).

The proof of a connection between the effect of cortisone on the increased permeability of the synovial membrane in rheumatoid arthritis and the amount of hyaluronidase inhibitors is lacking.

CLINICAL MATERIAL AND EXPERIMENTAL METHODS

The material consisted of 7 patients, 18—70 years old, with rheumatoid arthritis. A total of 9 knee joints with exudate were examined in these 7 patients. The exudate of the knee joints had persisted for 3—72 months. Before and during the investigation the patients were confined to bed. As a rule 50 mg of cortisone-acetate (2 ml cortone "Ciba"), was injected intra-articularly every day. There was exudate in both knee joints in 2 patients. In each of these cases one knee joint was used as a control and the other was simultaneously supplied with cortisone-acetate intra-articularly.

For laboratory examination 5—15 ml exudate were aspirated each time and in most cases daily. All of the cases were followed till exudate could no longer be obtained in the required amount.

The exudates were centrifugated immediately after aspiration from the joints and the viscosity was then determined at once with an Ostwald viscosimeter at 37° C. In most cases the exudates

ed by a grant from Konung Gustaf V:s 80- d.

were also incubated with hyaluronidase *in vitro* for 24 hours at 37° C and the viscosity was determined again. The results were stated in relative viscosity, according to the viscosity of *aq. dest.* at 37° C = 1.

The activity of hyaluronidase inhibitors was determined according to a turbidimetric method (Ekman 1953), which principally does not diverge from the method of Tolksdorff, McCready, McCullagh & Schwenk (1949), developed for determination of the activity of hyaluronidase. According to control experiments the amount of hyaluronic acid in the examined quantity of the exudate was so small, compared with the amount already present in the hyaluronidase-hyaluronic acid system, that it could be neglected.

The activity of hyaluronidase inhibitors in the samples was compared with the activity of a synthetic hyaluronidase inhibitor, viz. phloretin phosphate (Diczfalusy, Fernö, Fex, Högberg, Linderot & Rosenberg 1953). In the figures the activity hyaluronidase inhibitors per ml exudate is expre in γ phloretin phosphate.

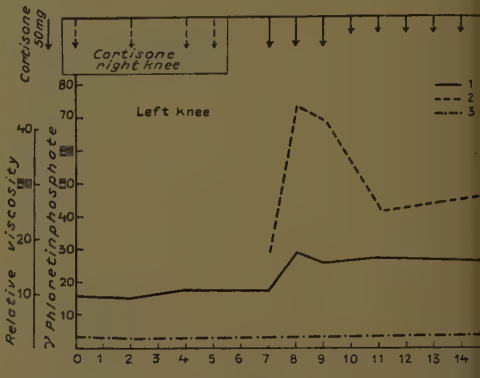


Fig. 2. Case with exudate in both knee jo Viscosity (1) and activity of hyaluronidase hibitors (2) before and during cortisone adminis tion. Viscosity after incubation with hyaluronidase (3).

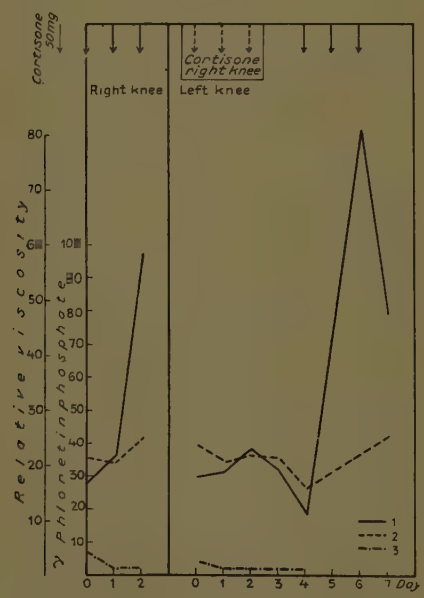
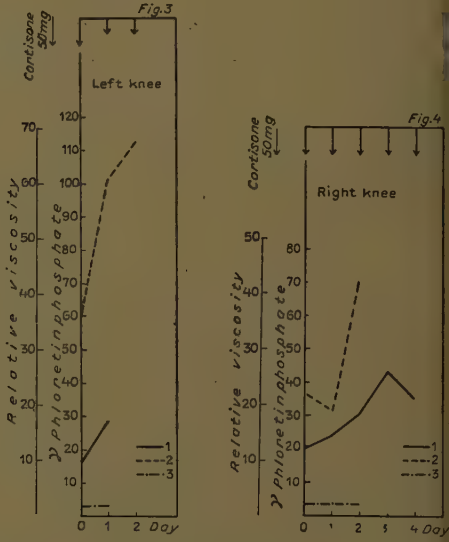
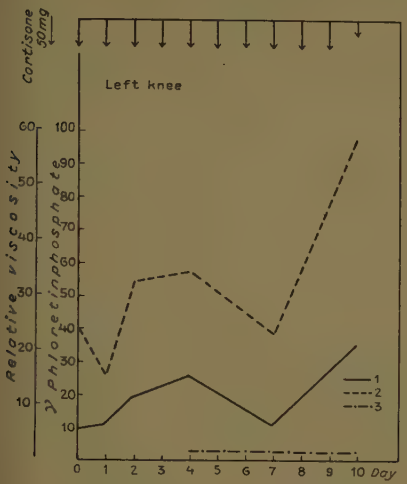


Fig. 1. Case with exudate in both knee joints. Viscosity (1) and activity of hyaluronidase inhibitors (2) before and during cortisone administration. Viscosity after incubation with hyaluronidase (3).



Figs. 3—4. Cases with exudate in one knee j Viscosity (1) and activity of hyaluronidase hibitors (2) before and during cortisone adminis tion. Viscosity after incubation with hyaluronidase (3).



5. Case with exudate in one knee joint. Visco- (1) and activity of hyaluronidase inhibitors (2) re and during cortisone administration. Visco- sity after incubation with hyaluronidase.

RESULTS

n both the control knee joints there was significant increase of viscosity (Fig. 2) or of activity of hyaluronidase inhi- rs (Fig. 1), when the other knee joints e supplied with cortisone.

ll the 7 knee joints supplied with corti- e-acetate showed an increase of the vity of hyaluronidase inhibitors (Figs. 5). An increase of viscosity was found ll the 6 knee joints in which the viscosity determined (Figs. 1—5). The effect d be registered in samples taken 24 or ours after the first cortisone injection. ncrease of the activity of hyaluronidase bitors and of viscosity was also obtained ontrol knee joints, when these were sup- l with cortisone-acetate after the admini- tion of cortisone to the other knee joint stopped (Figs. 1—2).

The maximal increase of viscosity reached approximately 5 times, and that of the activity of hyaluronidase inhibitors 3 times, the initial value.

In all samples of exudate the hyaluronidase reduced the viscosity to values about 2.0 (Figs. 1—5).

In all cases subjective improvement was obtained within one day or two, but only in the joint supplied with cortisone: decrease of pain in rest and movements. During the following days there was also a decrease of peri-articular swelling and exudate, and increase of mobility. The total amount of eosinophilic leukocytes was not reduced in the 2 cases which were controlled daily for 2 weeks. The erythrocyte sedimentation rate was influenced in none of the 7 cases.

DISCUSSION

In all knee joints supplied with cortisone there was increase of viscosity, increase of the activity of hyaluronidase inhibitors, decrease of the exudation and clinical improvement. In the 2 case with exudate in both knee joints there was no effect in the control knee joint during the period of investigation.

From these results it is obvious that cortisone injected intra-articularly has a local effect on the joint. The decrease of exudation may indicate that the permeability of the synovial membrane is reduced.

The first cortisone injections were followed by an increase of the viscosity and of the activity of hyaluronidase inhibitors considerably greater than the reduction of the exudate. Hence the result cannot be explai-

ned only by resorbtion of fluid from the exudate, but there must be a real increase of the activity of hyaluronidase inhibitors and of the viscosity.

The viscosity of all the exudates after incubation with hyaluronidase *in vitro* is found to be about 2.0. This indicates that the increase of viscosity found is due to changes in the hyaluronic acid. These changes may to some extent be the effect of inhibited depolymerisation of the hyaluronic acid. The observed increased activity of hyaluronidase inhibitors may be responsible.

SUMMARY

In joints with exudate in rheumatoid a ritis, intraarticular cortisone injection locally and is followed by:

1) a reduction of exudation probably to a decrease of the permeability of synovial membrane,

2) an increase of the viscosity induced hyaluronic acid,

3) an increase of the activity of hy ronidase inhibitors which may result i decrease of the depolymerisation of hy ronic acid.

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DILUTION CURVES AND ACCURACY IN PROTHROMBIN ESTIMATIONS DURING HEPATITIS AND DICUMAROL THERAPY

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The expression of the results of estimaons of the prothrombin complex in blood s been much disputed. Generally the pathogical clotting times have been converted to percentages of the normal combined fect of prothrombin and prothrombin conrsion accelerators (expressed as "per cent othrombin") in plasma by interpolation on curve obtained by plotting clotting times ainst relative concentrations of normal asma. However, it has not been generally alised that an important error is introduced this procedure, if the shapes of the dilun curves obtained with various pathogical or normal plasma samples do not rrespond to each other and to that of the ndard interpolation curve. In the present per a comparison is made of the shape of ution curves of a series of normal plasma mples and of samples obtained before and ring dicumarol therapy and in epidemic patitis. The significance of the variations evaluated and the accuracy of the prorombin determination is estimated, taking the factors into consideration.

METHOD AND PROCEDURE

Owren's method for the estimation of the comed effect of proconvertin and prothrombin wren 1949 a) was used as described by Astrup, illertz & Rud-Hansen (1951). Four batches thromboplastin and two batches of prothrombin-free ox-plasma were used during this investigation. The materials were stored in small tubes at —20° and the stability was controlled by repeated testing with samples of normal plasma.

Plasma dilutions. Blood samples were obtained by drawing 4.5 ml blood into 0.5 ml 2 per cent potassium oxalate. A 10 per cent stock solution was prepared from the plasma by diluting with buffer. The percentages in the present paper refer to the concentrations of this stock solution (= 100 per cent). The same dilution technique and the same pipettes were used in all experiments. Some of these experiments lasted several hours, but it was found that the reagents and plasma samples yielded identical results, at least within 4 hours at room temperature.

The determinations were performed by making one preliminary and two test clotting time estimations for each dilution of plasma. The mean value of the two last estimations was used. All determinations were performed by a technician with long experience in the procedure.

Dilution curves: When clotting times are plotted against relative plasma concentrations, a hyperbolic curve corresponding to equation (1) is obtained:

$$t = kc^{-a} \qquad (1)$$

In this equation t = time in sec.; c = relative concentrations of plasma; k and a = constants. The factor k expresses the prothrombin activity of the plasma sample, while a expresses the shape of the curve. By plotting the logarithms of clotting times against the logarithms of the relative concentrations, a straight surve is obtained according to equation (2):

$$\log t = -a \log c + \log k \qquad (2)$$

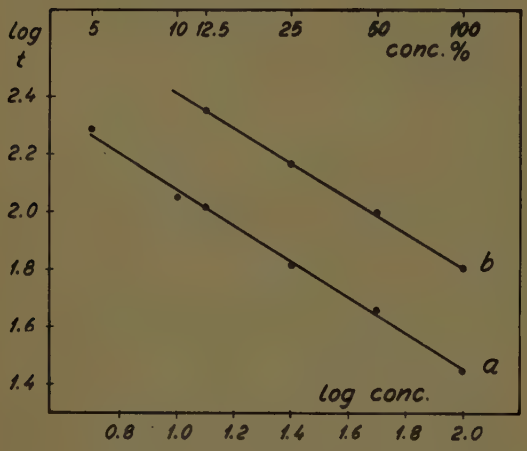

Fig. 1. Prothrombin dilution curves prepared with plasma obtained from the same patient a) before b) during dicumarol treatment. *Ordinate:* log. clotting times (sec.). *Abscissae:* Bottom: log. plasma concentration (per cent) . Top: plasma concentration in per cent of stock solution.

where a denotes the slope of the curve and $log\ k$ the position of the curve above the abscissa, expressed as the point of intersection with the ordinate axis in $log\ c = 0$ ($c = 1$). Two samples of different prothrombin activities but with the same value of a yield double logarithmic curves with identical slopes, but placed at a different distance above the abscissa (different values of $log\ k$) (Fig. 1). A mathematical treatment of the expression of prothrombin activity by double logarithmic curves has been given by Legler (1943). The technique used in the present study yielded curves corresponding to these functions. Only at very low prothrombin activities (3—5 per cent) were the logarithmic curves found to deviate from the straight line and to become steeper.

Nomenclature of new factors.

Most of the new factors involved in the conversion of prothrombin to thrombin appear to be identical. *Factors V* and *VI* (Owren 1944, 1947 a) correspond to *proaccelerin* and *accelerin* (Owren & Aas 1951, Astrup 1950) to *component A* and *labile factor* (Quick 1943, 1947), and to *plasma* and *serum Ac-globulin* (Ware & Seegers 1948). *Co-Factor* (Owren 1947 b) corresponds to *proconver* (Owren 1951), to *factor VII* (Koller, Loeliger Duckert 1951) and probably also to the *serum pr thrombin conversion accelerator* (de Vries, Ale ander & Goldstein 1949) and to the *κ-fact* (Sørbye, Kruse & Dam 1950). *Proaccelerin* a *proconvertin* are the terms used in the pres paper.

RESULTS

The accuracy of a single clotting ti determination was estimated by making series of determinations on dilutions of no mal plasma.Three series were run on di tions yielding mean clotting times of 33 43.0 and 89.0 sec. (Table I). The clotti times were converted into percentages interpolation on a standard curve and t deviations were estimated as percentages the corresponding plasma concentrations. is seen that, within the applied limits, t error increases somewhat with higher co centrations.

The accuracy of a single determination the slope of a dilution curve. Series of di tions of the same stock solution of norm plasma were prepared. Each series consist of 5 dilutions (100, 50, 25, 12.5 and per cent.)

Table I. *The accuracy of a single clotti time determination.*

Number of determ.	Results in sec.		Results in percenta	
	Mean values	Relative stand. dev. (%)	Mean values	Relative stand.
10	33.5±0.4	1 2	80±2.5	3.1
10	43.0±0.4	0.9	50±1.0	2.0
10	89.0±1.0	1.1	15±0.2	1.3

total of 50 dilutions were made and the tting times determined. The ten curves re drawn and the slopes estimated. The an value was —0.56; the standard devia- n was 0.01 and the maximal deviation 0.02.

The variation of the slope with different rmal plasma samples. Dilution curves re made with various normal plasma sam- s and with different batches of thrombo- stin. The determinations were performed different days within 2—3 weeks for ch group. In Table II the values are recor- d in the same sequence as they were per- rmed. The uniform distribution of the ues is evidence that the variations do not pend on a progressive qualitative change the thromboplastin. The calculated stan- rd and maximal deviations are also recor- d. It appears that the standard and maxi- l deviations are 3 to 4 times larger than corresponding deviations on a single termination of the slope of a curve as und above. Hence a definite variation in pes exists for different normal plasmas.

The variation of the slope with different thological plasma samples. Epidemic hepa- s. Dilution curves were constructed with plasmas from patients suffering from demic hepatitis and with prolonged pro- rombin times. In Table III the values are corded with the standard value determined described above for the corresponding ch of thromboplastin. It appears that the ues and the deviations are of the same gnitude as those found with different nor- l plasma samples.

Dicumarol treatment. In this material sam- were obtained from 10 patients suffering m acute coronary occlusion and treated h dicumarol. Samples were obtained from the same patient immediately before treat- ment and some days after the treatment had been instituted. In the cases where dicuma-

Table II. *Slopes of dilution curves of samples of normal plasma.*

Thromboplastin	A	B	C
Ox plasma	I	I	II
Sample Nr. 1	−0.60	−0.59	−0.67
2	−0.64	−0.57	−0.62
3	−0.66	−0.59	−0.63
4	−0.67	−0.56	−0.69
5	−0.58	−0.63	−0.64
6	−0.64	−0.56	−0.69
7	−0.60	−0.54	−0.68
8	−0.68	−0.59	−0.68
9	−0.62	−0.57	−0.70
10	−0.62	−0.60	−0.68
11	−0.64		
12	−0.62		
Mean (standard) value	−0.63	−0.58	−0.67
Standard deviation	0.04	0.03	0.02
Maximal deviation	0.05	0.05	0.05

Table III. *Slopes of dilution curves of various plasma samles obtained from patients with hepatitis.*

	T	O	a^{st}	PC	a^P	Deviation $a^P - a^{st}$
1.	C	II	−0.67	55 %	−0.69	+0.02
2.	—	—	—	60 —	−0.67	0.00
3.	—	—	—	60 —	−0.68	+0.01
4.	—	—	—	62 —	−0.67	0.00
5.	D	—	−0.67	56 —	−0.68	+0.01
6.	—	—	—	78 —	−0.70	+0.03
7.	—	—	—	42 —	−0.70	+0.03
8.	—	—	—	58 —	−0.71	+0.04
9.	—	—	—	60 —	−0.72	+0.05
10.	—	—	—	70 —	−0.67	0.00

T: thromboplastin. O: ox plasma. a^{st}: standard slope (mean slope of normal plasmas). a^P: slope of pathological plasmas. PC: prothrombin concentration.

Table IV. *Slopes of dilution curves of various plasma samples obtained (1) bef and (2) during dicumarol treatment.*

	T	O	a^{st}	PC		a^P		Deviation $a^P - a^{st}$		Deviati $a^P_1 - a$
				1	2	1	2	1	2	
1.	A	I	−0.63	56 %	11 %	−0.59	−0.61	−0.03	−0.05	0.02
2.	A	I	−0.63	100 %	28 %	−0.59	−0.60	−0.03	−0.04	0.01
3.	A	I	−0.63	44 %	17 %	−0.54	−0.56	−0.09	−0.07	0.02
4.	B	I	−0.58	85 %	21 %	−0.62	−0.62	+0.04	+0.04	0.00
5.	B	I	−0.58	90 %	42 %	−0.55	−0.57	−0.03	−0.01	0.02
6.	B	I	−0.58	90 %	35 %	−0.62	−0.65	+0.04	+0.07	0.03
7.	D	II	−0.67	100 %	27 %	−0.65	−0.70	−0.02	+0.03	0.05
8.	D	II	−0.67	84 %	31 %	−0.70	−0.72	+0.03	+0.05	0.02
9.	D	II	−0.67	110 %	46 %	−0.68	−0.70	+0.01	+0.03	0.02
10.	D	II	−0.67	110 %	62 %	−0.68	−0.70	+0.01	+0.03	0.02

For symbols: see Table III.

rol treatment was combined with an initial heparinization, blood was taken at least 4 hours after the last injection of heparin. During treatment only 4 dilutions of the stock solution of plasma (100, 50, 25 and 12.5 per cent) were estimated because of the considerable inaccuracy in the estimation of very prolonged clotting times and because of the deflection of the double logarithmic curve at very low prothrombin activities. For the same reason samples with prothrombin activity below 50 per cent but above 15 per cent of the normal were preferred. Within this interval the slopes of the curves could be estimated accurately. Similarly 6 dilutions (100, 50, 25, 12.5, 10 and 5 per cent) were prepared of the samples obtained before treatment to permit a comparison between the two sets of curves in the same region of clotting time. Two such curves are shown in Fig. 1. The results are recorded in Table IV. It is seen that the deviations of both sets of curves are uniformly distributed about the standard value and are not significantly larger than the deviations found with normal plasma samples except in single case (case 3). It is remarkable t the deviations between the curves obtai before and during treatment (dev. $a^P_1 -$ are so small, obviously smaller than deviations of both curves from the standa values (dev. $a^P - a^{st}$). This shows that individual variations are larger than th induced in a single individual by dicuma treatment.

DISCUSSION

Choice of method in clinical work: most methods the clotting times are conv ted into prothrombin concentrations by int polation on a curve obtained from the cl ting times of saline dilutions of norm plasma (e. g. Quick 1938, 1945). The d covery of the new clotting factors (above) was followed by the elaboration methods where proaccelerin was added the clotting mixtures in the form of plasm freed from prothrombin and proconver by adsorption (Rosenfield & Tuft 19 Owren 1949 a). However, the dilution c

vary in shape according to the diluent
d (Conley & Morse 1948, Biggs & Mac-
lan 1949, Frommeyer 1949). This is pro-
ly caused by the different relative con-
trations of the factors in the dilutions.
factors are diluted equally by addition
saline. Proaccelerin is kept constant while
thrombin and proconvertin vary, by addi-
n of adsorbed plasma. By the latter
ution technique, the known factors
varied in nearly the same way as they
e been found to vary during dicumarol
rapy and in non-malignant hepatitis
uick & Stefanini 1949, Owren 1949 b,
ller, Loeliger & Duckert 1951). This
umption is supported by the results pre-
ted here:

1) A variation in the slopes of dilution
ves for different normal plasmas was
nd. This suggests an individual variation
the ratio between the clotting factors.

2) Values of the same magnitude were
nd for slopes obtained with plasmas from
ients treated with dicumarol or suffering
m hepatitis.

3) The variation between samples from
same patient obtained before and during
umarol treatment was considerably smal-
than the individual variations.

Other relationships between the factors
y exist under special conditions, e. g.
amin K-deficiency (Sørbye, Kruse &
m 1950) and the shape of dilution curves
de with K-deficient plasma seems to dif-
from those described above (Larsen
52). However, in practical clinical work a
gle method should be preferred, which
ds the most correct results in the majority
cases. As dicumarol and hepatitis are by
the most common clinical causes of
hypoprothrombinemia, methods applying adsorbed plasma should be used.

The expression of results. This problem has been much disputed. See especially Larsen 1952), Conley & Morse (1948) and The Transactions of the Macy Foundation Conferences (1948, 1949, 1950). The clotting time of a normal plasma (100 per cent value) is often used as a control and the relation between the normal and pathological value has been expressed in various ways (Valentine, Reinold & Schneider 1941, Ziffren, Owen, Hoffman & Smith 1940, Quick 1939, Rapoport 1947). In most cases, however, various dilution curves are used (see above) Different thromboplastins may yield dilution curves of normal plasmas of different slopes (Conley & Morse 1948, Biggs & Macfarlane 1949 and the present paper). Hence a prothrombin-deficient plasma may yield different clotting times with different thromboplastins, even if the 100 per cent clotting times of normal plasma are identical. This shows that such a control is often useless. Also a universally applicable interpolation curve (Quick 1938) does not exist. A new standard curve should be prepared with every new batch of thromboplastin (Owren 1949 a) and this curve is only valid as long as the preparation is absolutely stable.

However, the present study shows that further precautions are necessary. Different normal plasmas yield curves of varying slopes. When plasmas differing in slopes are used to prepare standard curves with different preparations of thromboplastin, a certain clotting time will correspond to different percentages on the curves (Fig. 2). Therefore, a standard interpolation curve with the

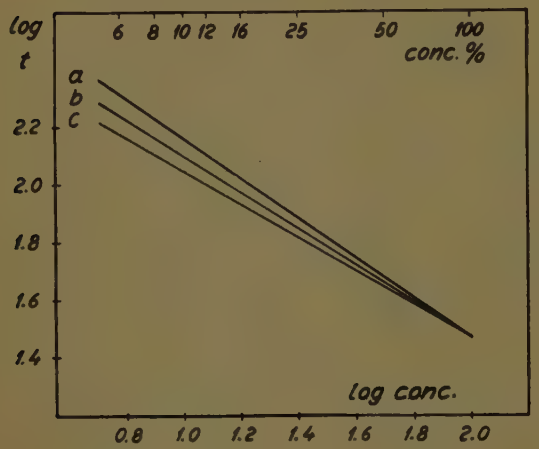

Fig. 2. Prothrombin dilution curves with identical 100 per cent clotting times and slopes of: a) — 0.68, b) — 0.63 and c) — 0.58. (Corresponding to mean value and limits for the slopes obtained with normal plasmas and thromboplastin A).
Symbols: see Fig. 1.

mean slope should be prepared by using a mixture of a number of normal plasmas. It is of great interest to examine what these percentage values really represent. By the described calculation the clotting times are expressed only in other terms introducing a correction for the variation of the reagents. However, these values are not a true expression of the concentration of prothrombin factors, as is generally believed. The conversion of clotting times to concentrations by means of a dilution curve is only possible in so far as the ratios between these factors remain constant. Therefore a method should be used which satisfies equation (1) in a large interval of concentration (Owren's method: 100 per cent—5 per cent of stock dilution 1:10). Different plasmas are only comparable in terms of concentrations if the double logarithmic dilution curves have equal values of *a* (see Fig. 1). A consid able variation in the values of *a* was fou for pathological as well for normal plas dilution curves. Therefore a true knowled of the content of clotting factors in differ plasmas cannot be obtained.

The accuracy of the determinations. T factors which determine the accuracy may arranged in two principal groups: A) error of the clotting time determinati proper, and B) the error involved in expression of the clotting times.

A. *The accuracy of the clotting ti determinations proper* depends on these f tors: 1) the number of replicates tested each determination, 2) the plasma dilut 3) the composition of the clotting mixt and 4) the technique, routine and skill the technician.

1) For practical reasons triplicates duplicates are run.

2) The minimum error probably around 0.5 sec. within a large range plasma concentration and clotting time. T a standard deviation of 0.4 sec was fo both for 43 and 33 sec. (50 per cent and 80 cent of the stock dilution 1:10) and Lar (1952) accurately determined the value 0.55 sec. (~ 0.4 sec for triplicates) for 1 sec. (100 per cent conc. of the stock dilut 1:3). The longer clotting times of high d tions again produce larger errors (Table The relative error on a clotting time basi rather large with undiluted plasma, decre to about a dilution of 1:20, but incre with still higher dilutions.

3) The addition of adsorbed plasma v proaccelerin and fibrinogen yields sho clotting times and a more defined coag tion and thereby a higher accuracy.

B. *The error involved in the expression the clotting times* depends on two factors: the plasma dilution and 2) the variation slope of the dilution curves.

1) The relation between plasma concentration and clotting times make a proper ution of the plasmas necessary. The ange in concentration which corresponds a certain change in clotting time, will increase rapidly with increasing plasma concentions (equation (1) and Fig. 1). Thus with vren's method (stock dilution 1:10) a standard deviation of 0.4 sec. corresponds to per cent at 80 per cent plasma concentration, to 1.0 per cent at 50 per cent, while 0.4 c. corresponds to less than 0.1 per cent at per cent (Table I). With undiluted sma (e. g. Quick 1945) a standard deviation of 0.5 sec corresponds at least to 20 per nt at 100 per cent plasma concentration king only prothrombin deficiences below per cent determinable. The optimal dilution is limited by the error of the clotting ne proper, and appears from a calculation the total accuracy (Table I).

2) The variation in slopes. A maximal viation of 0.05 was found between the pes for a number of normal plasmas able II). With one thromboplastin the an slope was found to be — 0.63. Therere curves with slopes of — 0.58 and — 8 (0.63 ± 0.05) may occur. The effect of s variation cannot be determined accurayy, but an impression may be obtained en these curves are drawn with identical 0 per cent clotting times (Fig. 2). A clotg time corresponding to 10 per cent on the an curve corresponds to 8 per cent and per cent respectively on the two other ves. If the variation in slopes is disregarded by the elaboration of standard curves, a (maximal) variation of 40 per cent of the interpolated results may occur for different thromboplastins in this clinically important region. This error is eliminated by the elaboration of standard curves with the mean slope for every new thromboplastin. However, the biological variation of the slopes of the pathological plasmas tested alone causes an unavoidable error of a similar magnitude. With a mean standard curve and a certain stock dilution, different plasmas with equal clotting times correspond to equal percentages. With another stock dilution (e. g. 1:5 or 1:20 instead of 1:10) neither clotting times nor percentages are equal. Thus two plasmas with slopes of — 0.58 and — 0.68 and with equal clotting times at the 1:10 stock dilution (100 per cent conc.) yield different clotting times at a 1:100 dilution (10 per cent conc.), and the corresponding percentages (on the mean curve) will be 8 per cent and 12 per cent respectively (fig. 2).

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CONCLUSION AND SUMMARY

1) A method was used (Owren) for the estimation of prothrombin, where adsorbed plasma was added to the clotting mixtures. Double logarithmic dilution curves were obtained for a number of normal persons and

from patients treated with dicumarol or suffering from hepatitis.

2) The mean values and the limits of the variation of the slopes of the curves were found to be of the same magnitude for normal and pathological plasmas. The slopes of curves obtained with plasma from a patient before and during dicumarol treatment did not differ significantly. Hence the use of adsorbed plasma yields correct results under these common clinical conditions.

3) The variation in slopes makes it impossible to obtain a true knowledge of the content of clotting factors in different plasmas.

4) The accuracy of the determinations was investigated. It was shown that the error caused by the unavoidable biological variation of the slopes is by far the most important and much larger than the methodical error. The influence on the accuracy of the varying activity of the thromboplastin samples may be eliminated by means of standard curves with the mean slope. The methodical error was found to be 1.3 — 3.1 per cent, when the results were expressed as percentages of a normal value.

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URINARY CALCIUM OUTPUT DURING TREATMENT WITH ACTH

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Cushing's syndrome is associated with overproduction of the so-called S hormone (Albright, 1942) which causes inhibition of the protein synthesis, involving also the osteoid tissue. Most often the osteoporosis is localized, most conspicuously in the vertebral column.

Analogously it was to be expected that such a state of osteoporosis would be aggravated by ACTH therapy, and hence the symptom has been included among the contraindications for ACTH therapy (Berglund, 1952, Kinsell, 1951). It is questionable, however, whether it be warranted to make such a far-reaching comparison between a chronic disease and often brief treatment with one of the hormones whose presence in pathological amounts gives rise to the development of Cushing's disease.

So far only relatively few investigations have been published on the calcium metabolism in man under treatment with ACTH.

Albright (1950) investigated the calcium excretion under ASTH treatment in 6 patients suffering from various affections (Paget's disease, panhypopituitarism, ovarian agenesia and acromegaly). Four of these patients showed a rise in the calcium output which Albright considered ascribable to impurity of the ACTH preparation (presence of pitressin) while two were tre ted with a purer preparation showed a f in the calcium output.

Luft & Sjögren (1950) examined 3 pa ents with rheumatoid arthritis under cor sone treatment for 3—4 weeks and foun on a constant diet, a rise in the urinary ca cium output whereas the fecal calcium e cretion decreased in two patients under th treatment and rose in one. Even though t clinical data given in these cases are ve brief, it is still evident that the two patien with a negative calcium balance befor during and after the cortisone treatme were confined to bed chronically, while t third patient — who showed calcium balan throughout the observation period — was and moving about with the aid of a walkin stick.

While most authors have found the seru calcium to be unaffected under treatme with ACTH, Friedberg (1952) demonstr ted a fall in serum calcium in younger pa ents. Hypocalcemic tetany also been de cribed (Roelsen, 1952). Boland & Headl (1950) have seen spontaneous fractur occur under protracted cortisone therap As both of their patients were diabetic however, the authors took the fractures these patients to be an accidental concidenc

Experimentally, in infantile rats, an inition of the osteoplastic activity has been monstrated while the osteoblasts appeared altered (Ducommon, 1950).

It would have been desirable to have studed the total calcium balance, but determination of fecal celcium was not practicable at time. Still, we think, it will be justible to draw some conclusions from variations in the urinary calcium output because is a common and established experience at arrest of the normal rebuilding of the nes — for instance, during immobilization manifests itself in an increased excretion calcium with the urine. So, if treatment th ACTH inhibits the osteoblast activity, a e in the urinary calcium output is to be pected. The prerequisite of any conclusion that during their observation period the tients have the same physical exercise and constant diet.

PATIENT MATERIAL

The urinary calcium excretion was examined in atients, three of whom had rheumatoid arthritis, postmenopausal osteoporosis, one essential hypoathyroidism, one thrombocytopenic purpura, and sarcoidosis. With the exception of Nos. 5 and who were kept in bed with active exercise, all patients were up. As the present examinations re made for orientation, all the patients were en ACTH only for a short time. The diet was same for all the patients except No. 1, who was en ordinary hospital diet without milk or milk ducts.

se 1 (FML, 31 446).

Woman, aged 56, with post-climacteric osteosis that has brought about roentgenographic ctures of the 9, 10 and 11 dorsal and 4 lumbar tebrae. Since 1949 ambulatory treatment with bon arc light, vitamin D, estrogenic and androic hormones — without improvement. The patient was up and moving about, on a standard diet with 100 mg calcium a day.

The curve shows the low urinary calcium output, varying with the diuresis. Towards the end of the ACTH treatment a slight rise in urinary calcium was seen, corresponding to an increase in the diuresis. Thus the calcium concentration has been fairly constant.

Case 2 (FML, 34 592).

Man, aged 76, with essential thrombopenia but no bone affection. He was up and about during the experimental period.

The curve shows a rise in the urinary calcium output during the ACTH treatment in spite of euphoria and lively motor activity.

Case 3 (FML, 31 651).

Woman, aged 56. In 1931, essential hypoparathyroidism was diagnosed. From 1941 she was treated with calciferol, without control of serum calcium. Since 1945, manifest damage to the kidneys. In 1949, admitted to the Finsen Institute, and calciferol was discontinued on account of metastatic calcifications. In 1951, readmitted to the Finsen Institute.

Serum calcium varying, 7.4—5.0 mg per cent. During the experimental period the patient was given calcium lactate 1.5 g, ferrotartrate 75 g and aluminum hydroxide 1 g daily. Before the experiment the calcium output was low, and it rose somewhat under the ACTH treatment, corresponding to the increase in diuresis, even though the calcium output rose less than the diuresis. This may be due to a fall in the concentration of ionized serum calcium, as a state of alkalosis developed, with beginning tetany.

Case 4 (FML, 35 198).

Woman, aged 51, with sarcoidosis localized to the skin, mucous membranes and bones, also universal halisteresis.

Decrease in the calcium output was seen during two periods of treatment with ACTH. Possibly this fall may be due to the circumstance that the previously immobilized distressed patient now moved more freely, but a specific ACTH effect on sarcoid granulomas in the bone cannot be excluded.

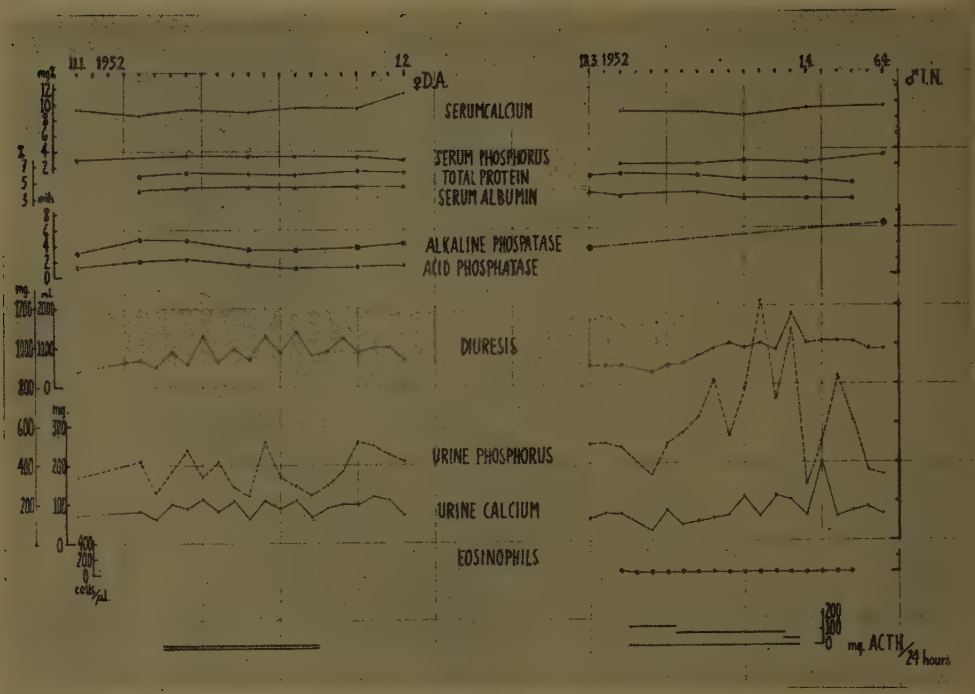

Fig. 1—2. (Case 1 & 2.)

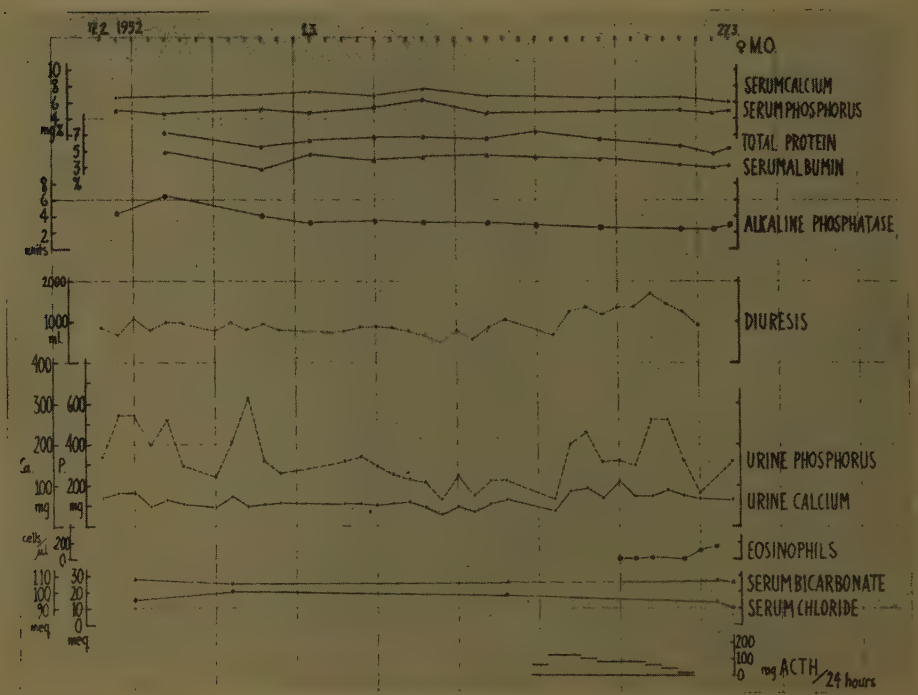

Fig. 3. (Case 3.)

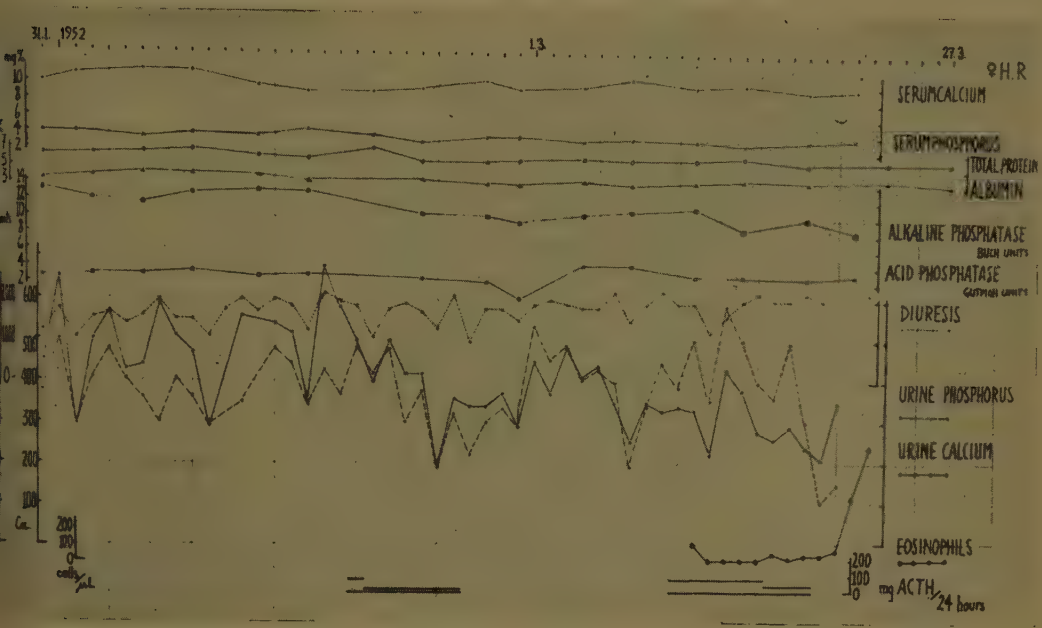

Fig. 4. (Case 4.)

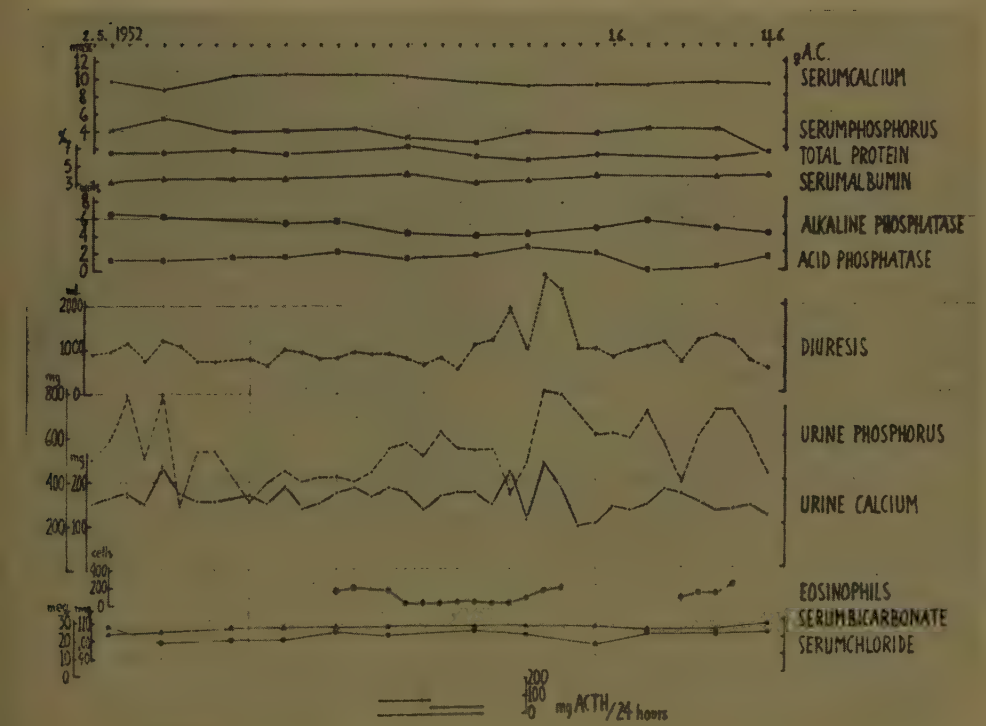

Fig. 5. (Case 5.)

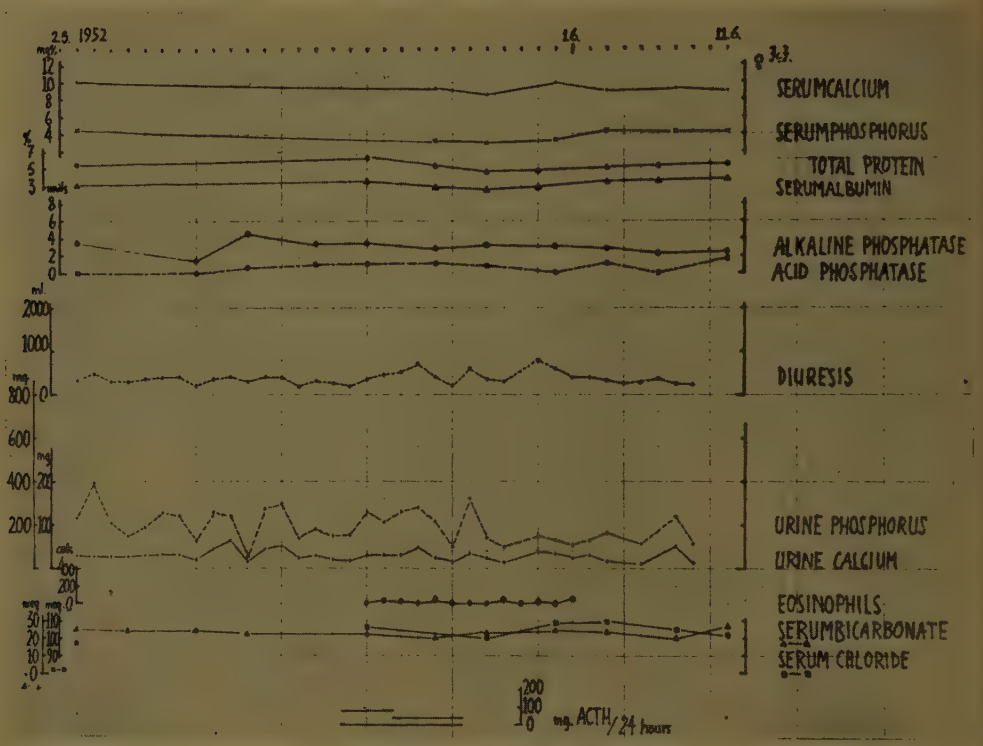

Fig. 6. (Case 6.)

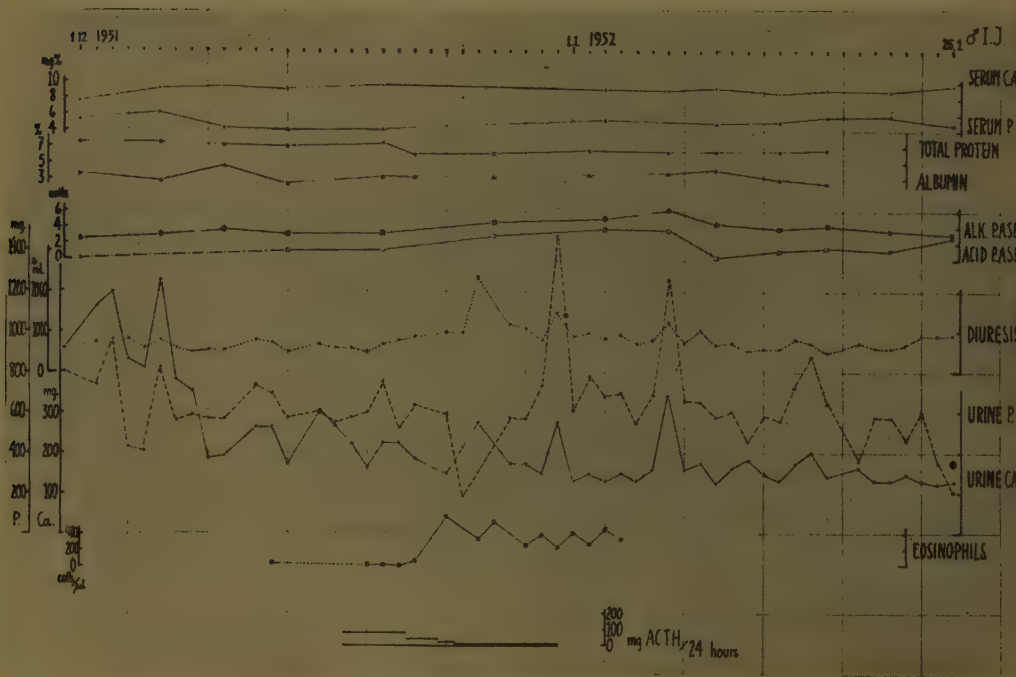

Fig.7. (Case 7.)

se 5 (FML, 35 895).

Woman, aged 52, disabled from progressive eumatoid arthritis. She has been suffering from lyarthritis for 20 years, and during the last year e has been confined to bed almost constantly on count of pain and subfebrilia, treated with physio- erapy, sanocrysin and hyperpyrexia without any rticular effect. She kept to her bed during the perimental period.

On May 12, exercise with active movements was stituted and continued under the ACTH treat- ent, which was commenced on May 19 and lasted 6 days.

se 6 (FML, 11 599).

Woman, aged 82, who had been suffering from ogressive rheumatoid arthritis through the last years. During the experiment she was treated er the same plan as patient No. 5. Neither in s nor in the preceding case was any change seen the calcium output under the ACTH treatment.

se 7 (FML, 35 002).

Man, aged 58, with rheumatoid arthritis and pro- unced universal halisteresis. He had been up for out one month and was given short wave dia- rmy and massage.

At the commencement of active movements (Dec. a very large calcium output was reduced to half, h an additional fall to a lower level under the CTH treatment. After discontinuance of this atment the calcium output kept at the same low el.

DISCUSSION

From the curves here presented it will be iced that in this experimental series there s no change in serum calcium under CTH treatment. Still, we have to reckon h a rise in the amount of ionized calcium responding to the fall in serum protein en the total calcium value remains con- nt, whereas the tendency to alkalosis will ng about a decrease in the amount of ioni- l calcium.

The urinary calcium output in our cases showed a rise in 3 patients corresponding to an increase in diuresis; 2 patients showed no rise in the diuresis nor in the calcium output; and 2 patients showed a fall in the calcium output during and after the ACTH treatment. We do not take the considerable fall in calcium output in these two patients to be a specific ACTH effect but perhaps it may result from the euphorizing and analgesic effect that brings about an increased motor activity in patients whose inactivity osteoporosis is due to pain. The rise in the calcium output here observed under the ACTH treatment must be said quantitatively to be of no significance to the course of an osteoperosis possibly present.

SUMMARY

A report is given of the results of daily urinary calcium determinations under ACTH treatment in 7 patients, three of whom had rheumatoid arthritis, one hypoparathyroidism with calciferol intoxication, one sarcoidosis, one post-menopausal osteoporosis and one essential thrombopenia. Prior to the experiments the urinary calcium output was low in five of these cases, three of the patients showed a rise in the calcium output corresponding to the increase in diuresis, and two showed no changes.

Two patients with a high urinary calcium output showed a marked fall in the calcium excretion which the authors attribute to an increased motor activity which more than counterbalances the possible osteoblast-inhibiting effect of ACTH.

All blood and urine examinations have been made at the Finsen laboratory (chief: Dr. phil. O. Henriques) to whom our thanks are due.

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THE EFFECT OF LIVER EXTRACT IN CULTURES OF MEGALOBLASTIC BONE MARROW

PRELIMINARY REPORT

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(Received for publication Nov. 27, 1952)

In tissue culture experiments on bone rrow from pernicious anemia patients in apse, Lajtha (1950) studied the effects various hemopoietic substances. He ob-rved an increased change of a megaloblas- into normablastic marrow after the addi-n of liver extract and folic acid.

Vitamin B_{12} was ineffective in this res-ct. This was confirmed by Franco & kun (1951) and Thompson (quoted by ngley, (1951). Our own experience with amin B_{12} (Cobione Merck in a concentra-n of 0.4 γ per ml of culture fluid) was in cordance with these reports. Biesele & rger (1950), however, observed an incre-e in the number of mitoses with vitamin $_2$ in 6 hour cultures of normal rabbit rrow.

Callender & Lajtha, (1951 a) thought, t this activity of liver extract was due to folinic acid content. In a later paper 951 b) they reported, that vitamin B_{12} is ive in bone marrow cultures only, when it combined with an "intrinsic factor" of tric or extragastric origin. They point that their work supports the original stle hypothesis. According to this theory extrinsic factor, which we now know is ntical with vitamin B_{12}, is transformed by the intrinsic factor of normal gastric juice into the active hemopoietic principle.

We thought it worth while to study the effect of a highly purified liver extract containing no folic acid or folinic acid. According to Wijmenga, Veer & Lens (1950) and Wijmenga (1951), vitamin B_{12} may occur in purified unautolyzed liver extracts as proteinocobalamine bound to a protein or polypeptide. In the same series of experiments this extract was tested after the addition of KCN. Then cyanocobalamine is produced, while the protein part of the proteinocobalamine is also left in the solution. Both preparations are effective in promoting the growth of *Lactobacillus leichmannii*.

In the control experiments folic acid, which is known to be active, and Tyrode solution were added to the medium.

Three criteria for the activity were used:

1) the decrease of pathological marrow cells,

2) the disappearance of young cells (promegaloblasts, proerythroblasts, basophilic megaloblasts and erythroblasts, myeloblasts and promyelocytes) and

3) the number of mitoses.

The method of culture was essentially that described by Osgood & Brownlee

Table I. *Per cent of the total number of cells.*

		Original suspension	Cultured with Tyrode		Cultured with Folic acid		Cultured with cyano cobalamine liver extract		Cultured wi proteino- cobalamine liver extrac	
			After 24 hours	After 48 hours	After 24 hours	After 48 hours	After 24 hours	After 48 hours	After 24 hours	After hou
Pat. K.	Young	39	27	6	19	7	18	5	19	
	Pathologic	52	36	20	24	20	28	9	31	2
Pat. B.	Young	19	19	20	11	6	12	8	21	
	Pathologic	60	44	38	29	23	26	24	29	2
Pat. G.	Young	35	25	5	16	7	19	6	17	
	Pathologic	52	47	36	44	26	44	34	45	3
Pat. K.W.	Young	36	19	2	15	4	10	3	15	
	Pathologic	60	33	12	27	14	25	14	26	1

(1937). At least 500 cells in 4 smears of each culture tube were counted. Each tube contained 2 ml of a cell suspension in 80 per cent pernicious anemia serum + 20 per cent Tyrode solution to which 0.25 ml Tyrode solution or 0.25 ml of diluted liver extract containing 0.8 γ of vitamin B_{12} or 0.25 ml of a solution containing 4 mg of folic acid was added.

From the data it appears, that folic acid is effective in promoting the disappearance of pathological marrow cells as well as the maturation of young cells. The same applies to both types of liver extract. A special feature of both liver extracts is the increased number of mitoses.

In these experiments very high concentrations of hemopoietic substances are used. If any conclusions may be drawn from these experiments, one may say that both liver extracts are active. From Callender & Lajtha's work, two possible explanations for the activity of liver extracts in cultures of megaloblastic bone marrow arise. Folic acid or a bound form of vitamin B_{12} might be active principles involved.

Folic acid or folinic acid are not pres in the liver extract used in this study.

As there is no difference in activity b ween the extract containing proteinoco lamine and the one with cyanocobalamine is probable that the presence of a prot conjugated form of cobalamine is not ess tial in order to make liver extracts active tissue culture studies.

It is difficult to explain these data.

Thymidine, if present, occurs only in sm amounts in the extracts used in this stu Thymidine was clinically inactive in per cious anemia in the dosage used by T Berg, Van Ravensteyn, Weyland, Brest Lens & Geerts (1949) who gave 3×10 and by Ungley (1949) who administe 48 mg. Only in the high dosage of Ha mann (200 mg daily) was thymidine cli cally active. Therefore it is improbable t thymidine is the active factor in these t

A possible explanation of these data may the occurrence in these extracts of an ive substance apart from the cobalamines.

Table II. *Frequency of mitoses.*

Original suspension:		
ultures	24/10 000	cells
After 24 hours:		
ultures with Tyrode solution ..	24/10 000	»
ultures with folic acid	29/10 000	»
ultures with liver extract containing cyanocobalamine	48/10 000	»
ultures with liver extract containing proteinocobalamine ..	31/10 000	»
After 48 hours:		
cultures with Tyrode solution ..	15/10 000	»
ultures with folic acid	14/10 000	»
ultures with liver extract containing cyanocobalamine	35/10 000	»
ultures with liver extract containing proteinocobalamine ..	41/10 000	»

is suggested accessory factor is not necesily hemopoietic in itself, but may be required for the different cobalamines to be effective.

If this hypothesis is correct, it might provide an explanation for the discrepancy between the observations of Horrigan et al. on the effect of vitamin B_{12} on megaloblasts, when instilled locally in the bone marrow cavity of pernicious anemia patients, and the data of Lajtha. In tissue cultures, the factor must be introduced in the medium if vitamin B_{12} is to be effective; in the *in vivo* experiments it may reach the marrow cell by the circulation.

ACKNOWLEDGEMENT

I wish to express my thanks to Dr. F. J. Keuning for his advice and his instruction in the technic of tissue culture and to the N. V. Organon, Oss, who made available the liver extracts used in this study.

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PRECAUTIONS AGAINST INFECTION OF PERSONNEL WORKING IN TBC. LABORATORIES

By H. CHR. ÉNGBÆK

From Statens Seruminstitut, Copenhagen, Denmark

(Received for publication Sept. 27, 1952)

During the last decade, the Tuberculosis Department of Statens Seriuminstitut has served also as a central laboratory for demonstration of tubercle bacilli in pathological products of human origin covering the entire country.

In 1951 we received altogether 79769 specimens, 11905 of which proved positive on cultivation, and about 50 per cent contained many tubercle bacilli.

Therefore it is necessary to take all reasonable precautions in order to protect the persons working in the laboratories against infection with T. B.

With the technique used in our laboratories (Holm & Lester, 1942), (Engbæk 1953) the easing-out of rubber stoppers from flasks and glases is one of the most dangerous steps in the procedure. Even if it was done carefully in front of a gas flame, droplets could spout in the room.

In order to prevent this, we now cover the stopper with a sterile dry rubber cap of thin translucent rubber. The cap used for flasks is 5.5 × ø 3.5 cm, for centrifuge tubes 7.0 × ø 2.5 cm (finger-stall). The centrifuge tube is 10 × ø 1.5 cm.

When the stopper is eased-out cautiously, the cap will catch any drops from the sample. The cap containing the stopper is placed immediately in a bucket for sterilization.

Fig. 1. Rubber cabs.

Fig. 1 shows the caps.

Experiments with coloured liquids show that the drops are caught in the top of cap, not in the lower part of it.

With such caps the fingers also get in contact with contaminated material dur this part of the work.

SUMMARY

During the handling of specimens in Tuberculosis Department we now cover rubber stoppers with rubber caps before stoppers are eased-out of the flasks glasses. The droplets are caught in the and will not spout in the room.

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Letters to the Editors

A METHOD FOR DETERMINATION OF THE OSMOTIC RESISTANCE OF ERYTHROCYTES BY MEANS OF THE ELECTRO-HEMOSCOPE

The methods for the determination of otic resistance of erythrocytes hitherto ilable for routine clinical use are very curate. The use of unbuffered solutions NaCl is a considerable source of error, he osmotic resistance changes with pH cobs & Parpart 1931; Ponder 1948). thermore the ocular determination of the mencement and completion of hemolysis mewhat uncertain, and that of 50 per cent olysis impossible.

he most exact method seems to be the surement of supernatant hemoglobin, ressed as a percentage of the maximum unt that can be liberated (Ponder 1948). this method seems to be too complicated routine clinical use.

ome preliminary investigations have been e to find out whether the "electro-hemo-e" described by Loeschcke and Wever 51) could be used for determining osmo-esistance in a way simple enough for cal use yet at the same time sufficiently rate.

he method is based on photoelectric ting of unhemolysed erythrocytes in a s of hypotonic buffer solutions in the oscope, and analysis of the resulting e. The salt medium used was an isotonic *phate* buffer of pH 7.3 (von Muralt 1948). It was diluted to a series of hypotonic solutions with a tonicity difference of 0.02. The red blood cells were washed three times and diluted 1 :500 in the phosphate solutions. A part of each solution was further diluted with the same solution for measurement with the hemoscope. The rest was centrifuged, and the amount of supernatant hemoglobin measured in a Beckman spectrophotometer as check on the degree of hemolysis.

Comparison of the hemoscope curve and a hemolysis curve obtained by estimating the optical density of the supernatant fluids showed that it was quite easy to obtain a hemoscope curve equivalent to about three-

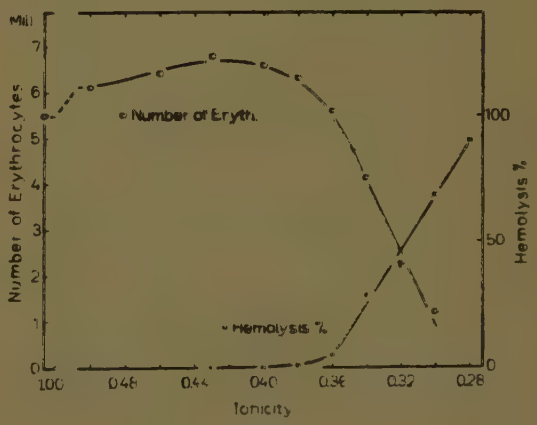

Fig. 1.

quarters of the hemolysis curve, including the starting point and 50 per cent hemolysis (Figure 1, 3½ hours after mixing). The former rises to begin with. The commencement of hemolysis seems to correspond to the highest point of the hemoscope curve. The point of 50 per cent hemolysis is the one which corresponds to half the number of unhemolysed red cells, as counted in isotonic solutions. This method gives higher osmotic resistance values than current clinical methods due to buffered salt medium.

A full report will be published later.

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March 30, 1953.

Martin Zade-Oppe

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A NEW APPARATUS FOR COLUMN CHROMATOGRAPHY

INTRODUCTION

The apparatus to be described is simple l adaptable, making use of standard glass nts, and enables adsorbed substances to eluted without disturbing the column, and collection of eluates in distinct fractions ile continuing suction.

CONSTRUCTION

As may be seen from the photograph, the paratus consists of a stout wooden stand which the glass parts are mounted by ans of Terry clips. The column-tubes are erchangeable, having a standard B 19 cone the base, and the B 19 socket which holds column-tube forms the centre of a T-piece, h a tap, A, A', on either side of the socket. ch limb of this T-piece is fused through obtuse angle to another T-piece, the upper b of which has a tap, B, B', opening to the and the lower limb is extended down ough a B 24 cone. From the top of each hese B 24 cones a tube passes at an angle wash-bottle fashion), to form the two notomous branches of a Y-piece, each with ap, C, C', at the centre. The main limb of Y is connected by pressure-tubing to a ss tube which passes through the base of stand, and is, in turn, connected with lter-pump.

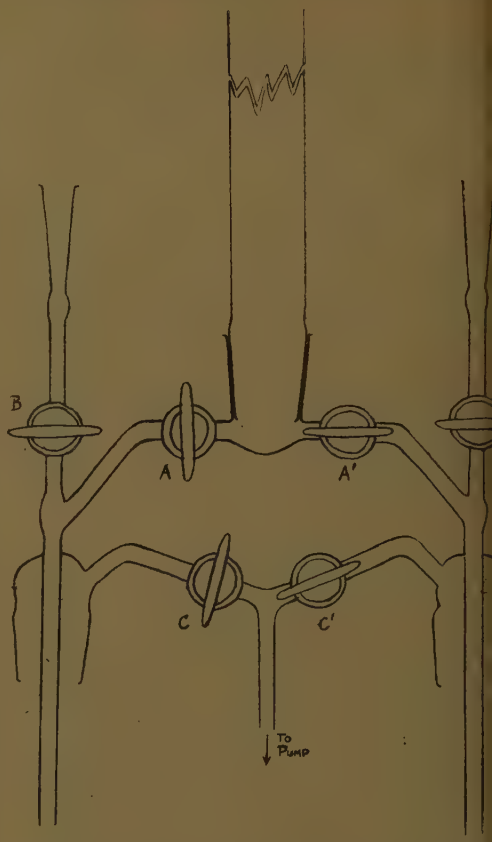
Flat-bottomed flasks, usually of 500 ml acity and with B 24 necks are fitted on to B 24 cones on each side of the apparatus, are supported by adjustable tables from base of the stand, clamped in position by ed knobs.

TECHNIQUE

After fitting on the receiver flasks, the left-hand side of the apparatus is closed off by turning the taps, and the right-hand side opened except the tap B' opening to the air.

The column is packed with the adsorbent, and if packed as a slurry in the solvent, the solvent runs down into the right-hand flask.

When the chromatogram is started, the same side flask is used as receiver until elution, when the bands begin to move. As soon as the first band approaches the base of the column, the right-hand side of the apparatus is rapidly closed and the left-hand side opened to the pump. The first band eluate is then collected in the left-hand receiver. Meanwhile, the tap B' on the right-hand side is opened to the air momentarily, and the flask changed. This side is now ready for the collection of the next eluted band, when the procedure just described is reversed. In this way, all the bands may be collected as eluates in labelled flasks. If two bands should be very close together in their passage down the column, it may be necessary to run the eluates of these two bands separately as chromatograms on another adsorbent.



DISCUSSION

The apparatus has found particular application in the purification and identification of porphyrins. (Kennedy and Vevers 1953 a, b.) In porphyrin purification, large columns of 40 cm × 6 cm were used, with Brockmann's alumina, silica-gel, and magnesia Grade III (Nicholas 1951). In identification, smaller columns 35 cm × 2 cm were used, with magnesia Grade III and Whatman Cellulose powder.

ACKNOWLEDGEMENTS

The author is much indebted to Mr. J. Hatfield of the Department of Physiology, who made the glass parts of the apparatus, to Mr. O. Thornburn of my laboratory who made the wooden stand, to Mr. John Gray of the Department of Fore Medicine for the photograph.

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February 20, 1953.

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A PHOTOELECTRIC MICRO METHOD FOR THE DIRECT TITRATION OF CALCIUM IN SERUM WITH ETHYLENEDIAMINE TETRACETATE

Since Schwarzenbach *et al.* (1946) introduced the direct titration of alkali earths especially calcium — with ethylenediamine tracetate (EDTA) with purpurate as indicator, several authors have described the e of this principle for the determination of lcium in serum, obtaining values in good reement with those of the Kramer & Tisll method (Greenblatt & Hartman, 1951, uckley, Gibson & Brotolotti, 1951, Elliot, 52, see also Smeets, 1952). However, the sual estimation of the end point is not sharp d values of higher accuracy than ± 0.5 mg r cent calcium have not been obtainable in is laboratory. In the following, two proceures for the accurate spectrophotometric gistration of the end point will be described, abling the determination of calcium in rum with high accuracy within a couple minutes.

The maximal light absorption of the calm-purpurate complex in 0.1 n NaOH was und at 505—510 mμ whereas the maxium absorption of purpurate in the absence calcium was around 550 mμ. Absorption rves during the titration with EDTA owed that all curves passed 550 mμ (the sobestic point" according to Schwarzench) with the same constant extinction lue whereas the disappearance of the red lor during the titration was accompanied a decrease of the extinction at 505 mμ til the end point was reached. Therefore light from the titration vessel falls on two otocells and a color filter selecting the 5 mμ range is placed in front of one of e cells and a filter of 550 mμ in front of the other, a deflection of the galvanometer needle will be seen as long as a color change at 505 mμ occurs — when the end point is reached the nedle is steady.

The technic was as follows: 8 ml 0.1 n NaOH together with 0.2 ml 20 mg per cent ammonium purpurate (B.D.H., the solution not older than 3 days) and 0.4 ml serum or calcium solution were pipetted into a 3 cm cell (Pulfrich cell) and placed in a Hellige photoelectric absorptiometer (old model) with a double photocell (Differentialzelle S. 60, Firma Lange) in front of which two Bausch and Lomb interference filters (505 and 550 mμ) were placed in a black frame. The photocells were connected + to + and a Cambridge spot galvanometer (external resistance 450 ohm, 6×10^{-9} amp. per mm scale) was adjusted to give an aperiodic deflection of 20—40 scale units for a complete titration.

The calcium was titrated with 0.001 m ethylenediamine tetracetic acid disodium solution (anal reag.). A standard curve for the interpolation of the found values of EDTA was constructed by titrating 0.4 ml of a 5—10 and 15 mg per cent calcium solution. The 10 mg per cent solution was titrated daily after good mixing of the EDTA solution.

The values were about 0.4 mg per cent calcium lower than those for the Kramer & Tisdall method and still 0.4 mg per cent lower for ashed serum. The correct values for serum are thus about 0.8 mg per cent lower than found with the usual methods using precipitation with oxalate as also found

by Smith *et al.* (1950) using flame photometric and spectrophotometric methods. The standard error of the method was ± 0.04 to 0.05 mg per cent calcium. When using 0.2 ml serum the error was somewhat larger.

The second method for the registration of the end point was to titrate the solution in test tubes or cells of a diameter of 15—20 mm in a photoelectric absorptiometer at a wave length of 505—510 mμ. (Coleman, Jr. or Beckman, B., with test tubes have been used.) The extinction values are noted ar registered on a millimeter paper. The e point is interpolated between the two slop of the curve obtained just before and aft the end point — the former being sharp bent upwards, the latter being flat a straight. This technic is somewhat more tir consuming than the first method describ but requires no special apparatus with t exeption of a usual photoelectric absorpti meter.

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Central Laboratory,
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June 8, 1953.

Jörgen Lehmann.

Book Review

ecent Advances in Clinical Pathology. Second Edition. General Editor S. C. Dyke. 561 pp. J. & A. Churchill Ltd., 104 Gloucester Place, London, W. 1.

The General Editor writes in the Fore-ord that as long as progress is made in edicine, there will be a need for periodical views. These will facilitate keeping up to te in the various fields of clinical pathology well as refreshing our previous knowledge. The book is divided into four main sec-ons: bacteriology, biochemistry, hematology d histology with an additional section on boratory design and equipment. The book the product of more than 30 contributors, ch an expert in his field, and satisfies the ed as stated by the General Editor in the oreword.

The subject matter is presented clearly d is well arranged, so that any particular bject may be readily found. The presenta-on is brief, which is a great advantage in e opinion of the reviewer, but not so brief at important details are omitted. In the apter "The Laboratory Control of Anti-agulant Therapy", however, the reviewer otes with regret the omission of Owren's ethod for determining the prothrombin ntent of plasma. This method has been idely used in Scandinavia ever since 1947, d in the reviewer's opinion affords a more liable basis than the Quick method for termining dicumarol dosage in individual ses.

It is not possible to review all of the 31 apters in detail. The reviewer was parti-larly impressed by the chapters on Hemo-obinometry, Hemolytic Anemia and Paper Chromatography, but anyone who is interested in following the progress of clinical pathology will gain by reading these reviews.

Both the General Editor and the Section Editors have done creditable jobs.

K. Ö.

Second Annual Report On Stress. Hans Selye and Alexander Horava. Acta, Inc. Medical Publishers, Montreal, Canada. 526 pp. 1952.

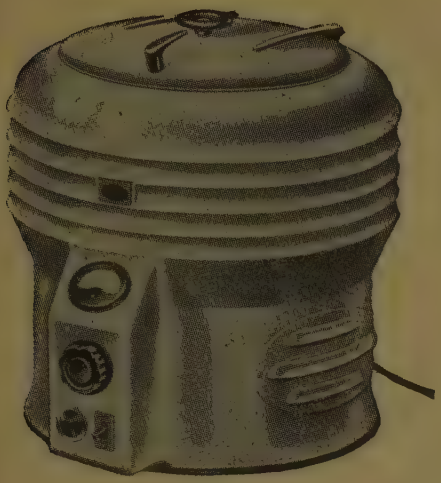
The first annual report has been reviewed recently in this journal. (Vol. 5, No. 1, 1953.)

This second report differs from its predecessor in that all discussions and descriptions are cut down to the most relevant points. The printing shows great economy, drawings published earlier are now reduced in scale, so is the text which serves as introduction to some of the topics listed under special physiology and pathology of stress. There are only minor alterations in the target and stressor lists of Selye, *inter al.* serology has been replaced by capillary permeability and inflammation. 13 pages are devoted to Selye's defence against criticisms and objections to the stress concept. Well over 5000 references are listed and ably indexed. Selected illustrations are found in an atlas covering 16 pages.

The second report fulfills every demand on an extensive classified index, containing pertinent facts from the literature, as well as observations made at Selye's laboratories during 1952.

For workers interested in the vast field of stress and adaptation mechanisms, the second report seems as indispensable as the first one.

O. J. M.



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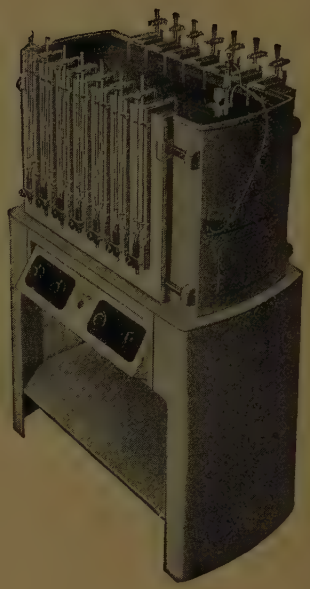

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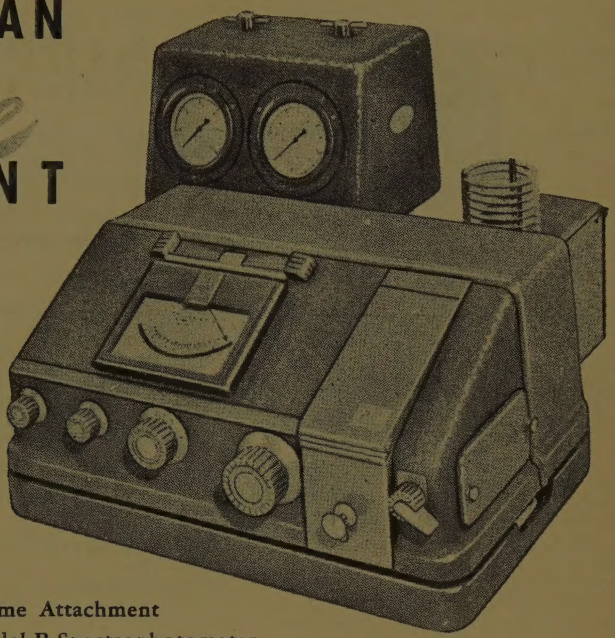

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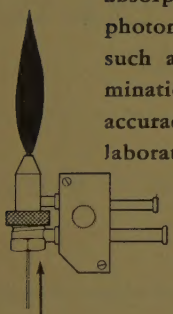

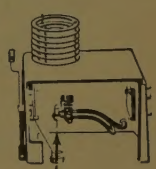

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CONTENTS

GÖSTA TUNEVALL: The antipneumolysin reaction and its clinical application 109
C.-B. LAURELL: The diurnal variation of the serum iron concentration 118
U. S. VON EULER, A. LUND, A. OLSSON, AND PH. SANDBLOM: Noradrenaline and adrenaline in blood and urine in a case of phaeochromocytoma 122
S. RADNER: Method for recording aortic pressure pulses 129
F. VOGT LORENTZEN: A simple method for the determination of functional residual air volume 132
TAGE ASTRUP: The toxicity of synthetic heparin substitutes 137
G. W. G. BIRD: The significance of auto-agglutinins 143
B. OLHAGEN AND A. SVANBORG: Attempts to produce experimental lipid nephrosis with tridione 146
K. GRETTE: Micromethod for the determination of calcium and magnesium in serum 151
L. ERIKSEN: Paper chromatography of porphyrin pigments 155
B. LAURENT AND E. A. NIKKILÄ: Studies on experimental proteinuria I 158
M. SUNDBERG: Sensitized clotting time as a clinical method 172
B. EKMAN, S. THUNE, AND E. TRUEDSSON: Investigation of viscosity and hyaluronidase inhibitors in joint exudate after intra-articular cortizone injections 175
S. MÜLLERTZ: Dilution curves and accuracy in prothrombin estimations during hepatitis and dicumarol therapy 179
P. J. DRAGSTED AND N. HJORTH: Urinary calcium output during treatment with acth 188
H. O. NIEWEG: The effect of liver extract in cultures of megaloblastic bone marrow 195

Technique and practical problems

H. CHR. ÉNGBÆK: Precautions against infection of personnel working in tbc. laboratories 198

Letters to the editors

M. ZADE-OPPEN: A method for determination of the osmotic resistance of erythrocytes by means of the electro-hemoscope 199
G. Y. KENNEDY: A new apparatus for column chromatography 201
J. LEHMANN: A photoelectric micro method for the direct titration of calcium in serum with ethylenediamine tetracetate 203
BOOK REVIEW 205

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